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Elucidating the LPS Modification Repertoire of *Pseudomonas aeruginosa*

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Elucidating the LPS Modification Repertoire of *Pseudomonas aeruginosa*

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2015

Dedication

To my amazing parents, Janine and Lenny Nowicki, for your unending love and support. You are both my heroes and have helped shape me into the person I am today. And to my grandmother, Alberta Nowicki, for inspiring me to always strive for the best and never give up.

Acknowledgements

First, I express my sincere gratitude toward my mentor, Dr. Stephen Trent. His guidance, support, brilliant ideas and input have helped me to grow as a scientist. Although the learning will never stop, his advice, wisdom, and constructive criticisms will remain ingrained in my brain throughout my career and fuel me to keep going. Stephen, I truly cannot thank you enough for your attention and most of all your patience throughout the years. I feel tremendously lucky and grateful to not only have had the opportunity to work for you, but to know you as a person.

I would like to thank my lab mates, past and present, for all of their time and help throughout my graduate training. Grad school would have been impossible without my lab "family". I have a particular appreciation for all of the help from David Giles, Brittany Needham, and Carmen Herrera. I also appreciate the many detail-oriented conversations during lunch, most often relating to food, that I had regularly with certain lab mates—these lighthearted conversations kept me sane during some stressful times!

To my committee members, Marvin Whiteley, Brian Davies, Jason Upton, and Mary Jo Kirisits—thank you for your advice, feedback, and support.

To my family, particularly my parents Janine and Lenny, my sister Alli, and my Grandma Alberta; even from all the way back in New Jersey you have all been my support system throughout this crazy journey. I love you all and cannot thank you enough for your love and support.

Finally, to my love, Adam—you have been my rock, my sounding board, my voice of reason. Thank you for always helping and listening to me, even though you're going through this process yourself.

Elucidating the LPS Modification Repertoire of *Pseudomonas aeruginosa*

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The University of Texas at Austin, 2015

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Gram-negative bacteria enhance their survival in harmful environments by outer membrane remodeling, particularly at the lipid A moiety of LPS. We recently identified a functional ortholog of the lipid A kinase, *lpxT*, in *Pseudomonas aeruginosa*. $LpxT_{Pa}$ is unique from previously characterized LpxT enzymes in that it is able to phosphorylate both lipid A phosphate groups as well as generate a novel 1-triphosphate species. Low Mg^{2+} results in modulation of $LpxT_{Pa}$ activity and is influenced by transcription of lipid A aminoarabinose (L-Ara4N) transferase ArnT, which is induced when Mg^{2+} is limiting (Nowicki *et al., Mol Micro,* 2014). We have also revealed the identity of a functional phosphoethanolamine (pEtN) transferase, $EptA_{Pa}$, in *P. aeruginosa*, and the first report of pEtN-modified lipid A in this organism. $EptA_{Pa}$ adds pEtN strictly to the non-canonical position of lipid A. Transcription of $eptA_{Pa}$ is regulated by Zn^{2+} via the ColRS two-component system, contrasting from EptA regulation in enteric bacteria such as *Salmonella enterica* and *Escherichia coli.* Further, although L-Ara4N modification readily occurs at the same site of pEtN addition under several environmental conditions, Zn^{2+}exclusively induces pEtN addition to lipid A and downregulates transcription of the L-Ara4N transferase gene (Nowicki *et al., Mol Micro,* 2015). The existence and specificity of these modification enzymes suggests that coordinated regulation of *P. aeruginosa* outer membrane remodeling occurs to permit adaptation to a changing environment.

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Chapter 1: Introduction

1.1 THE GRAM-NEGATIVE BACTERIAL CELL ENVELOPE: A PROTECTIVE BARRIER

1.1.1 Structure and properties of the Gram-negative cell envelope

The bacterial cell envelope is a complex, dynamic structure that serves as a protective barrier against perturbations in the surrounding environment. The defining feature of Gram-negative bacteria is their cell envelope structure which consists of two lipid bilayers. While the Gram-negative inner membrane is composed entirely of phospholipids in both leaflets of the bilayer, the outer membrane is asymmetrical with phospholipids forming the inner leaflet, and lipopolysaccharide the outer leaflet (Fig. 1.1A)(1). Divalent cations such as Mg^{2+} and Ca^{2+} intercalate between LPS molecules to help stabilize the outer membrane (2). The outer membrane is the first line of bacterial defense against environmental stressors, serving as a protective barrier to prevent binding and uptake of toxic molecules such as cationic antimicrobial peptides (CAMPs) (2). Study of bacterial surface structure and its impact on cell survival in potentially deleterious conditions is critical in order to understand an organism's ability to adapt and persist.

The major component of the outer membrane, lipopolysaccharide, interfaces with the environment and can be remodeled as the cell's surroundings change to offer the bacterium greater protection against harmful conditions. Lipopolysaccharide is composed of three distinct domains: a lipid A anchor, a core sugar region, and an outer polysaccharide known as O-antigen (Fig. 1.1A)(1). Lipid A is first synthesized through the conserved nine enzyme Raetz pathway. The resulting molecule has a β-1',6-linked di-glucosamine backbone that is phosphorylated on either side as well as fatty acylated (Fig. 1.1B). After lipid A is synthesized, a short chain of sugars is added at the inner

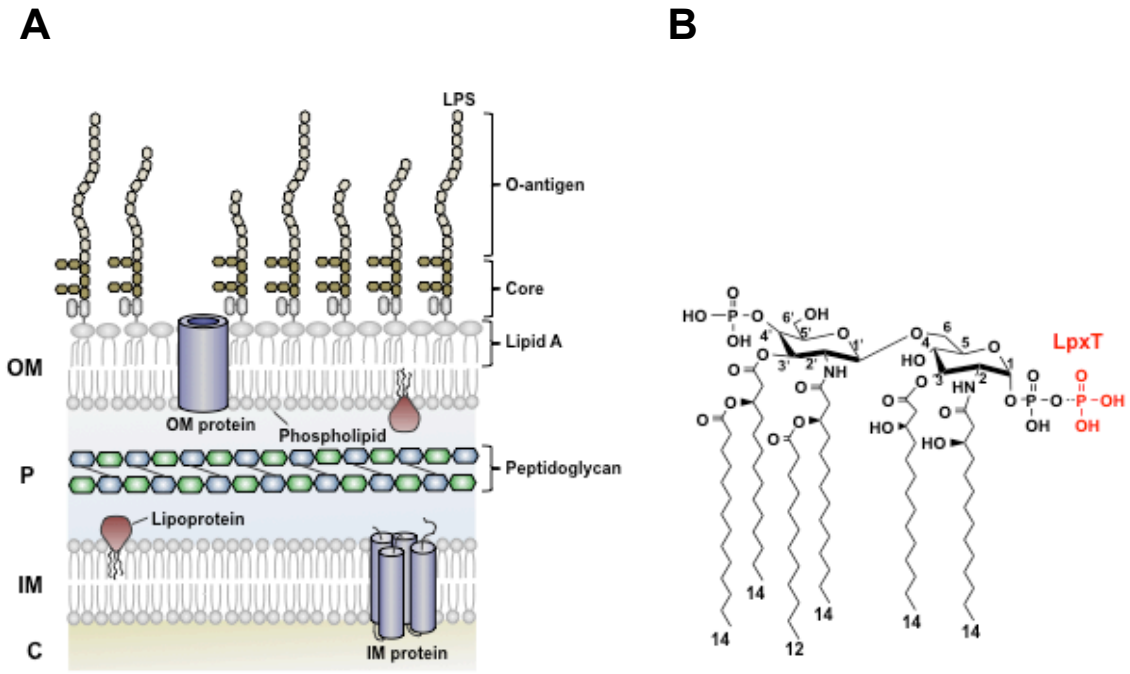

Figure 1.1: Structures of the Gram negative cell envelope and the lipid A region of LPS.

A) Diagram of the Gram-negative cell envelope; the cytoplasm (C), inner membrane (IM), periplasm (P), and outer membrane (OM) are depicted along with membrane proteins as indicated. The lipid A, core, and O-antigen domains of lipopolysaccharide (LPS), which forms the outer leaflet of the outer membrane, are indicated. **B**) Structure of the canonical hexa-acylated, *bis*-phosphorylated lipid A in *E. coli* synthesized via the Raetz pathway. Addition of a second phosphate group at the 1-position by the kinase LpxT, which composes approximate one-third of *E. coli* lipid A species in standard laboratory growth media, is indicated in red.

leaflet of the inner membrane, forming the core. The core region is often substituted with non-sugar residues, such as amino acids, phosphate groups, or ethanolamine moieties that can impact surface charge and antimicrobial resistance (3). After its synthesis, lipid A-core is then flipped to the periplasmic face of the inner membrane by the MsbA transporter, and O-antigen is subsequently conjugated to the molecule (1). O-antigen consists of a long chain of repeating sugar subunits, and varies greatly between species and even strains within the same species. Due to this great variability and strain-specific interaction with antibodies, O-antigen is used to serotype bacterial strains (3).

Although core and O-antigen regions of LPS are undoubtedly critical for host infection, the majority of LPS immunostimulatory activity is due to lipid A. The lipid A domain is often referred to as endotoxin as it is a potent activator of the innate immune system (4). Mammalian cells contain Toll-like receptor 4 (TLR-4), a pattern recognition receptor that binds to bacterial lipid A along with the help of LPS binding protein and the co-receptors myeloid-differentiation factor 2 (MD-2) and CD14 (5). Binding initiates a downstream, pro-inflammatory signaling cascade culminating in the production of NF-κB, which then induces genes that promote clearance of the pathogen (2, 6). Resulting products include cytokines, CAMPs, and immunostimulatory molecules (7). Although the immunostimulatory properties of lipid A can lead to bacterial clearance, overwhelming amounts of endotoxin in the system can lead to septic shock, characterized by capillary leakage, hypotension and vascular coagulation that leads to multiple organ failure and in some cases death (7–9). In addition to its role in activating the immune system, lipid A is also the prime target of CAMPs and alterations in divalent cation concentrations that can disrupt the outer membrane and cause lysis (2, 10). Although interactions with the host immune system and antimicrobial peptides are extremely

harmful for bacteria, Gram-negatives have evolved a number of strategies to modify their lipid A structure to promote their survival (2).

1.1.2 The importance of studying lipid A in the opportunistic pathogen, *Pseudomonas aeruginosa*

Pseudomonas aeruginosa inhabits soil and water sources and is known for its intrinsic tolerance to antimicrobials and potentially toxic contaminants such as heavy metals (11, 12). It is also a formidable opportunistic pathogen frequently acquired in healthcare facilities due to its persistence in sinks, showers and many non-aquatic abiotic surfaces (11, 13, 14). Once inside a human host *P. aeruginosa* thrives in a variety of tissue types resulting in acute skin, eye, and burn wound infections (13). Perhaps most notably, however, are chronic *P. aeruginosa* infections that persist within the lungs of cystic fibrosis (CF) patients for years and are recalcitrant to most antimicrobial treatment (11, 15). CF disease is genetically characterized by extensive mutation of the transmembrane conductance regulator or *CFTR* gene that encodes a chloride channel. Mutation of this gene results in malfunction of the channel and causes abnormalities in airway surface fluid. Ultimately, this leads to a reduced ability to clear inhaled microbes. *Pseudomonas aeruginosa* has specific affinity for the CF lung and adapts to thrive in the respiratory mucosa (16, 17).

Due to its adaptability, *P. aeruginosa* infections are extremely difficult to treat and are often debilitating or even fatal (13). The dynamic outer membrane of Gram-negatives like *P. aeruginosa* is a major contributor to its persistence, and the ability to remodel lipid A is one facet of this. Although a hexa-acylated lipid A is initially synthesized in *P. aeruginosa* as in enteric bacteria (Fig. 1.2A), specific lipid A structures are found in *P. aerugionsa* depending on the source of the isolate. The lipid A

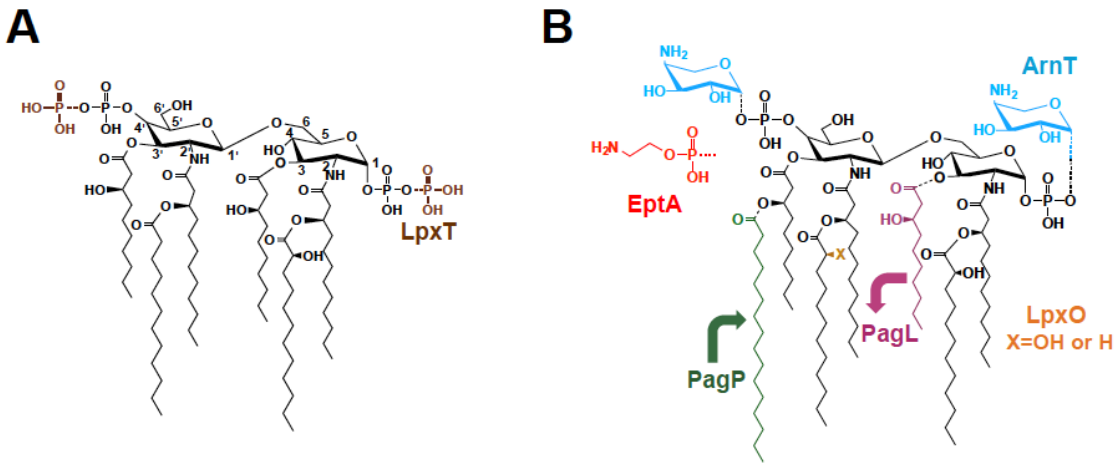

Figure 1.2: *P. aeruginosa* lipid A structure with and without modifications.

A) Canonical, hexa-acylated, *bis*-phosphorylated lipid A structure is shown in black. Phosphorylation of the lipid A phosphate groups by the LpxT kinase, which occurs in standard growth media, is indicated in brown. **B**) Possible modifications to *P. aeruginosa* lipid A are indicated in color, including addition of a palmitate chain by PagP (green), removal of the 3-hydroxydecanoate acyl chain by PagL (pink), hydroxylation of the C12 secondary acyl chain(s) by LpxO (orange), addition of L-Ara4N at the lipid A phosphate groups by ArnT (blue), and pEtN addition by EptA (red).

acylation pattern characteristic of *P. aeruginosa* from chronic CF lung and airway infections is a stronger TLR-4 stimulant than the lipid A typically found associated with acute infection or environmental isolates. This toxicity significantly contributes to lung damage, one of the hallmarks of CF disease that leads to worsened patient prognosis (6). Other modifications to *P. aeruginosa* lipid A lead to greater antimicrobial resistance, another characteristic attribute of *Pseudomonas* (18, 19). Chapters 2 and 3 will provide a detailed analysis of two previously uncharacterized *P. aeruginosa* lipid A modification enzymes, and provide evidence that various lipid A modification enzymes in this organism are precisely coordinated in response to specific environmental conditions. Since *P. aeruginosa* lipid A remodeling has been previously shown to play an important role in this organism's virulence, examination of these new modifications and their potential biological role could add significant value toward our understanding of *Pseudomonas* infection.

1.2 LIPID A MODIFICATIONS

The product of the Raetz pathway is a hexa-acylated, *bis*-phosphorylated lipid A molecule as shown for the model organism *Escherichia coli* (Fig. 1.1B) and for *P. aeruginosa* (Fig.1.2A). Although this hexa-acylated lipid A is initially synthesized in nearly all Gram-negatives, elaborate lipid A modification systems exist that are responsible for the diversity of lipid A molecules produced by different bacterial species (1, 2). Changes can be made to the acylation pattern of lipid A, as well as to the lipid A phosphate groups. While some of these modifications occur constitutively in certain bacterial species, others occur exclusively in response to specific environmental conditions and are tightly regulated.

1.2.1 Acyl chain modifications and effects

Alterations to the lipid A acyl chains can affect the toxicity of lipid A, as well as bacterial resistance to CAMPs. One such change is the addition of a palmitate (C16:0) acyl chain via the enzyme PagP in organisms including *E. coli, S. enterica* and *P. aeruginosa* (Fig. 1.2B) (19, 20). Palmitate is cleaved from phospholipids in the outer leaflet of the outer membrane, allowing the cell to reduce the phospholipid content while simultaneously modifying the lipid A structure (20). PagP is an outer membrane β-barrel protein with its active site at the outer surface of the cell (21). Transcription of *pagP* is induced under conditions that compromise outer membrane integrity and increase the phospholipid content in the outer leaflet, such as limiting Mg^{2+} concentration (20, 21). Lipid A palmitoylation has been shown to provide resistance to certain CAMPs including the synthetic α-helical peptide C18G generated from human platelet factor IV (19, 22). The altered packing of acyl chains is thought to affect movement of the peptide through the membrane (22). Additionally, the hepta-acylated lipid A generated by palmitate addition in *E. coli* and *S. enterica* is significantly reduced in toxicity and is unable to induce production of the inflammatory mediators TNFα and NF-κB (23).

Removal of an acyl chain can also occur via the outer membrane β-barrel lipase, PagL, in organisms including *P. aeruginosa* and *S. enterica* (2, 24). In *P. aeruginosa,* this enzyme removes the hydroxydecanoate acyl chain at the 3 position (Fig. 1.2B) (7, 25). While removal of this acyl chain has no effect on CAMP resistance, it does affect TLR-4 stimulation. Deacylated lipid A purified from *E. coli* expressing *S. enterica* PagL showed a 30-100 fold decrease in TLR-4 stimulation compared to unmodified, hexa-acylated lipid A (26). Penta-acylated lipid A species characteristic of *P. aeruginosa* laboratory, acute infection, and environmental isolates are also far less stimulatory than hexa-acylated species commonly found within the CF airway (6).

One last alteration that can be made to lipid A acyl chains in organisms such as *S. enterica* and *P. aeruginosa* is hydroxylation by the enzyme LpxO. A single, inner membrane LpxO enzyme exists in *S. enterica* that transfers a hydroxyl group to the 2-position acyl chain (27). In *P. aeruginosa*, two LpxO enzymes exist that hydroxylate each of the secondary lauryl chains (Fig. 1.2B) (3). LpxO activity is oxygen-dependent, and although active under normal laboratory conditions in both organisms, it can also be induced when Mg^{2+} concentration is limiting in *S. enterica* (28). LpxO enzymes in *P. aeruginosa* have yet to be fully characterized, and the biological role of this modification remains a mystery. A study in *S. enterica* however has revealed that in this organism LpxO, along with the virulence and stress-related periplasmic protein VisP, has a key role in pathogenesis. Without LpxO, *S. enterica* cells exhibit decreased survival within macrophages and virulence within a murine *in vivo* systemic infection model (29).

1.2.2 Phosphate group modifications and effects

The phosphate groups of lipid A can be modified with several different moieties, which can alter the net charge of the membrane and affect its stability. In *E. coli,* the outer membrane kinase LpxT transfers a phosphate group from undecaprenyl pyrophosphate (Und-PP) strictly to the 1-phosphate group of lipid A. This results in regeneration of undecaprenyl phosphate (Und-P), an important precursor involved in the biosynthesis of bacterial membrane components, while simultaneously forming *tris*-phosphorylated (1-diphosphate) lipid A (30). *Tris*-phosphorylated lipid A composes approximately one-third of *E. coli* lipid A grown in standard laboratory conditions, thus this modification does not seem to require any particular inducing condition (Fig. 1.1B)

(30). Recently, we identified an LpxT ortholog in *P. aeruginosa* that unlike *E. coli* LpxT, can modify either the 1 or the 4′ phosphate groups under normal growth conditions (Fig. 1.2A). We also found that *P. aeruginosa* LpxT can add a third phosphate group to the 1 position resulting in a unique 1-triphosphate species, although this lipid A species has only been seen in the absence of the 4′ phosphate group (31). The characterization of LpxT will be described in further detail in Chapter 2.

Positively charged moieties can also be added to mask the anionic phosphate groups of lipid A. This serves to increase resistance to CAMPs, as these positively charged peptides act by binding to the lipid A phosphate groups to mediate their entry into the cell, thereby displacing the stabilizing Mg^{2+} and Ca^{2+} ions (32). The inner membrane enzyme ArnT transfers the cationic sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the 1- and 4'-phosphate groups of *E. coli, S. enterica,* and *P. aeruginosa* lipid A, to name a few examples (Fig. 1.2B) (33–35). Transfer occurs in the periplasm using the lipid undecaprenyl phosphate-α-L-Ara4N as a donor (36). The *arnT* gene, formerly known as *pmrK*, is part of the *pmrHFIJKLM* operon that along with the unlinked *pmrE* locus together are involved in the biosynthesis and addition of L-Ara4N to lipid A (37). Substitution of L-Ara4N at one or both lipid A phosphate groups is known to be an important modification for CAMP resistance (38), and is characteristic of constitutively resistant strains (39).

Another positively charged group, phosphoethanolamine (pEtN), is added preferentially (or exclusively) to the lipid A 1-phosphate group by the inner membrane pEtN transferase, EptA, in a number of Gram-negatives including *E. coli, S. enterica* and

Helicobacter pylori (2, 40, 41). While pEtN addition plays a more minor role in CAMP resistance in *E. coli* and *Salmonella enterica* (40, 42, 43), pEtN modification of *H. pylori* lipid A results in a dramatic increase in resistance to the cyclic CAMP polymyxin (44). Mutants of the *eptA* ortholog, *eptC*, in *Campylobacter jejuni* have a significant colonization defect in both chick and mouse infection models, and are drastically reduced in motility (45, 46). In addition to modifying lipid A, EptC transfers pEtN to the flagellar rod protein FlgG; this modification is necessary for proper assembly of the flagellum and thus motility in *C. jejuni* (46). Further, *Neisseria gonorrhoeae* unable to modify lipid A with pEtN demonstrates a marked fitness advantage in both mice and human infection (47). We have recently identified a functional EptA enzyme in *P. aeruginosa* that strictly modifies lipid A at the 4' phosphate group (Fig. 1.2B)(48). This modification is described in detail in Chapter 3.

In organisms such as *Fransicella novicida* (an environmental strain related to the pathogen *F. tularensis*) and *H. pylori,* the lipid A phosphate groups can be removed entirely by the action of the inner membrane phosphatases LpxE and LpxF. LpxE selectively cleaves the lipid A 1-phosphate group (41, 49), while LpxF targets the 4′ phosphate group (50, 51). In *F. novicida*, LpxF has been shown to have additional specificity for tetra- and penta-acylated lipid A substrates (50). Removal of either phosphate group reduces the ability of lipid A to activate TLR-4 by a factor of 10^3 (52). In addition to decreasing the toxicity of lipid A, removal of either or both phosphate groups also results in a drastic increase in polymyxin resistance, with 4′ dephosphorylated lipid A showing greater resistance than the 1-dephosphorylated species

(50, 51). Removal of the 1-phosphate group of *H. pylori* by LpxE is also a requirement for pEtN addition to lipid A, and significantly increases polymyxin resistance (41, 44). Further, removal of either or both phosphate groups is critical for *H. pylori* colonization of gastric mucosa, as LpxE, LpxF or double mutants are impaired in host colonization in a murine model (51).

1.2.3 Lipid A phenotypes characteristic of acute and chronic P. aeruginosa infections

While many of the lipid A modifications described thus far often only occur in response to specific environmental cues, *P. aerugionsa* lipid A from CF infection isolates commonly has constitutive modifications (15). Studies comparing lipid A from chronic CF infection isolates to those from patients with acute conditions (such as bronchiectasis, blood, ear or urinary tract infections), environmental isolates, or laboratory-adapted strains identified CF-specific lipid A species. CF lipid A contains palmitate and often L-Ara4N, modifications that are normally regulated and typically only found during specific environmental conditions (i.e. low Mg^{2+}, subinhibitory CAMP concentrations) (Fig. 1.3) (34). Further, approximately one-third of *P. aeruginosa* lipid A isolated from CF patients, particularly from adults with severe lung disease, showed loss of PagL activity, and is thus hexa-acylated (25, 53). Modification of this hexa-acylated lipid A with a palmitate chain results in a unique, hepta-acylated structure (6). Environmental and acute infection isolates on the other hand are generally penta-acylated and were not found to contain palmitate or L-Ara4N medication (Fig. 1.3) (53).

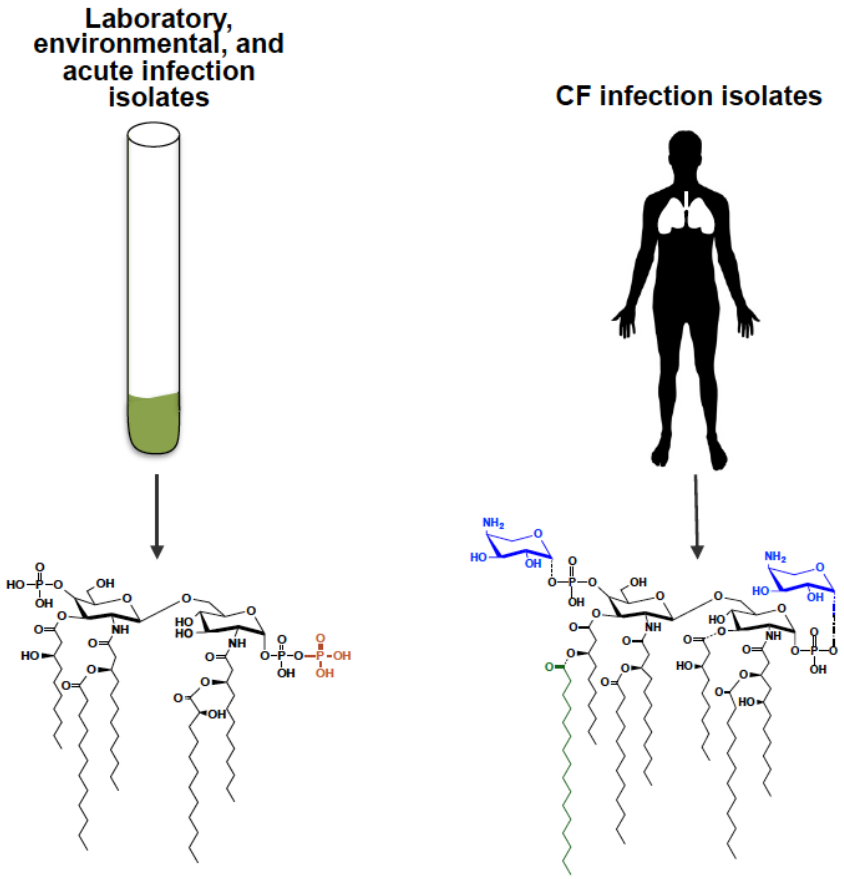

Figure 1.3: Lipid A structures characteristic of *P. aeruginosa* strains isolated from different source.

The penta-acylated, *bis* or *tris*-phosphorylated lipid A structure most often found in laboratory-adapted, environmental, and acute infection isolates of *P. aeruginosa* is depicted on the left side. Phosphorylation by the kinase LpxT (brown) is shown. On the right, the hexa- and hepta-acylated lipid A structures commonly found in CF infection isolates are shown. CF infection isolates are often modified with an additional palmitate acyl chain (green), and L-Ara4N moieties (blue), as indicated.

The lipid A structure characteristic of *P. aeruginosa* CF isolates has been shown to promote inflammation and CAMP resistance within the lung. The palmitoylated, hexa-acylated lipid A typical of *P. aeruginosa* CF isolates is highly immunostimulatory and reacts strongly with TLR-4. In contrast the penta-acylated lipid A structure of *P. aeruginosa* laboratory, environmental, and acute infection isolates can be >100-fold less stimulatory (6, 34). Lipid A isolated from *P. aeruginosa* CF strains also shows significant enhancement in the production of IL-8, an inflammatory mediator whose production contributes to increased lung inflammation and damage (34). In addition to the affect on toxicity, CF-specific lipid A species are also innately more resistant to CAMPs due to their modification with L-Ara4N and palmitate. Many CF isolates have been found to be highly resistant to colistin (also known as polymyxin E), a cyclic CAMP that is commonly used as a second line treatment of CF bacterial infections (54). The lipid A of these highly resistant strains is consistently modified with L-Ara4N and often palmitate, highlighting the clinical importance of lipid A remodeling (53).

1.3 REGULATION OF LIPID A MODIFICATIONS

1.3.1 Transcriptional Regulation

Transcription of lipid A modification enzymes is often induced through two-component system signaling (2). Signal transduction occurs when a bacterial sensor kinase autophosphorylates in response to an external stimulus and then transfers this phosphate group to a response regulator protein. The resulting conformation in the

response regulator promotes DNA binding at target promoters to alter gene expression (55). Response regulators can also be dephosphorylated by their cognate sensor kinases allowing further control over gene expression (56). The systems and mechanisms responsible for regulation of lipid A modification are best understood in the model organisms *E. coli* and *S. enterica*. In both of these organisms, the PhoPQ system regulates *pagP* transcription in response to low Mg^{2+} concentration as well as subinhibitory CAMP concentrations (20). In *S.* enterica, *pagL* transcription is also activated in a PhoPQ-dependent manner (7). In addition to *pagP* and *pagL*, the PhoPQ system induces LpxO-mediated hydroxylation of *S. enterica* lipid A, although this modification can occur at a basal level even in standard growth conditions (28)(Fig. 1.4).

A second two-component system, PmrAB, regulates transcription of *arnT* and *eptA* in both *E. coli* and *S. enterica* (42). In these organisms, PmrAB is activated in response to mildly acidic pH (57), excess ferric iron (58, 59), and other conditions that are not as well characterized including excess Al^{3+} (60) and Zn^{2+} (61) (Fig. 1.4). In addition to activation of PmrAB upon sensing these signals, this system can also be indirectly activated when the PhoPQ system is induced in both *S. enterica* and *E. coli* (62, 63). This connection of PhoPQ and PmrAB is possible via the action of the PmrD protein (62). PmrD interacts post-transcriptionally with PmrA and prevents its dephosphorylation to keep it active (Fig. 1.4) (62, 64).

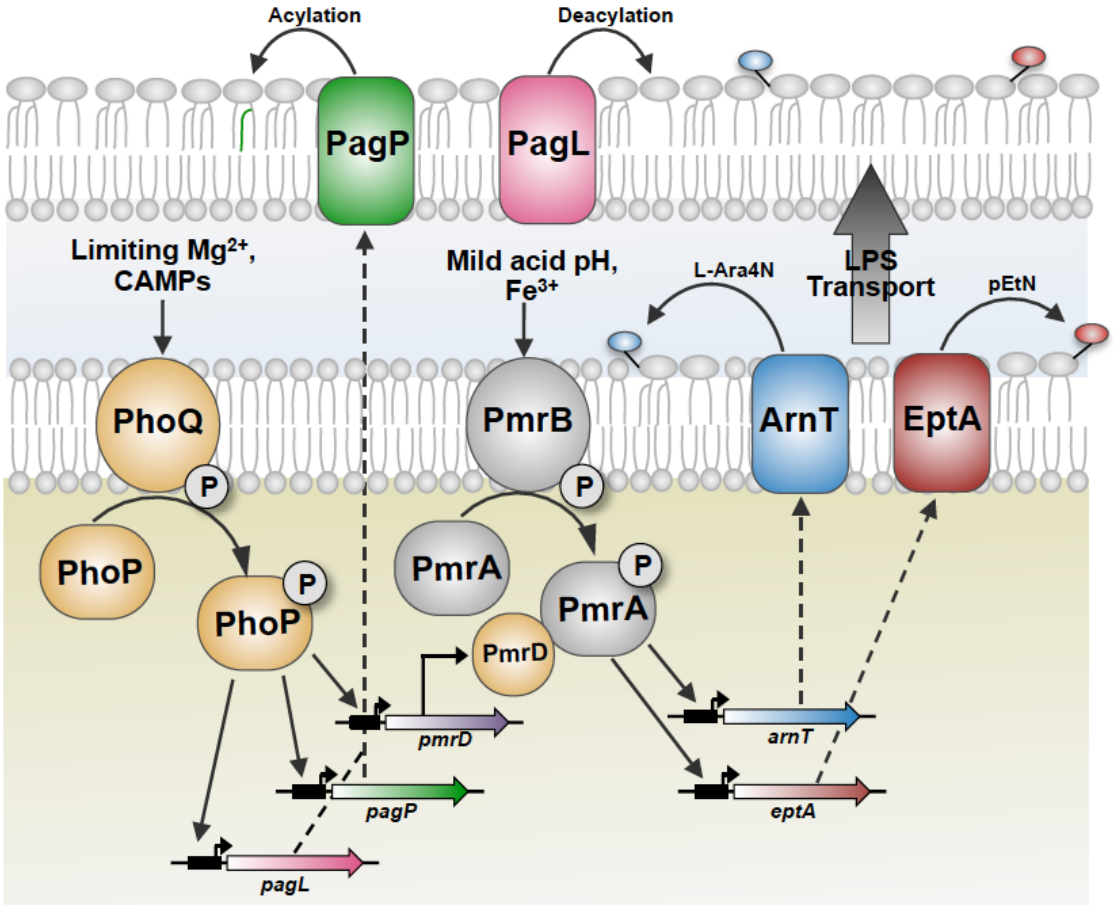

Figure 1.4: Transcriptional regulation of *S. enterica* lipid A modifications by the PhoPQ and PmrAB two-component systems.

In *S. enterica,* the PhoPQ two-component system responds to limiting Mg^{2+} as well as subinhibitory concentrations of CAMPs. This system induces expression of the *pagL* and *pagP* lipid A modification genes, as well as activating transcription of the PmrD protein. PmrD interacts with PmrA, keeping it phosphorylated and thus activated even when the PmrAB system is not activated. Through this PmrD connector protein, PmrA can activate transcription of *arnT* and *eptA* when Mg^{2+} is limiting or when CAMPs are present. The PmrAB system can also activate *arnT* and *eptA* gene expression independently of PhoPQ, in response to a variety of environmental stimuli including mild acidic pH and excess Fe^{3+}.

The two well-conserved two-component systems PhoPQ and PmrAB also play a major role in modulating lipid A modification gene expression in *P. aeruginosa,* although there are a few notable differences between *P. aeruginosa* and the model organisms discussed. While the PhoQ sensor of *S. enterica* can both activate and repress PhoP activity by phosphorylation or dephosphorylation, respectively, *P. aeruginosa* PhoQ might play a greater role in repressing PhoP activity (65). Although not yet determined, it is plausible that a second kinase capable of activating PhoP under Mg^{2+} limitation exists (65, 66). The PhoP response regulator activates *pagP* and *arnT* transcription in response to limiting Mg^{2+} (Fig. 1.5)(19, 67). In *P. aeruginosa, arnT* transcription can also be induced by the PmrAB system upon sensing either limiting Mg^{2+} or subinhibitory CAMP concentrations (Fig. 1.5) (67, 68). Although *P. aeruginosa* PagL is constitutively active in both laboratory strains and many CF isolates of *P. aeruginosa*, its activity in acute clinical and environmental isolates can be enhanced in the presence of limiting Mg^{2+} (25). It is possible that either the PhoPQ or PmrAB systems are involved in regulating *pagL* transcription, although this has never been shown. To date, whether or not a connection exists between the PhoPQ and PmrAB systems in *P. aeruginosa* remains to be determined.

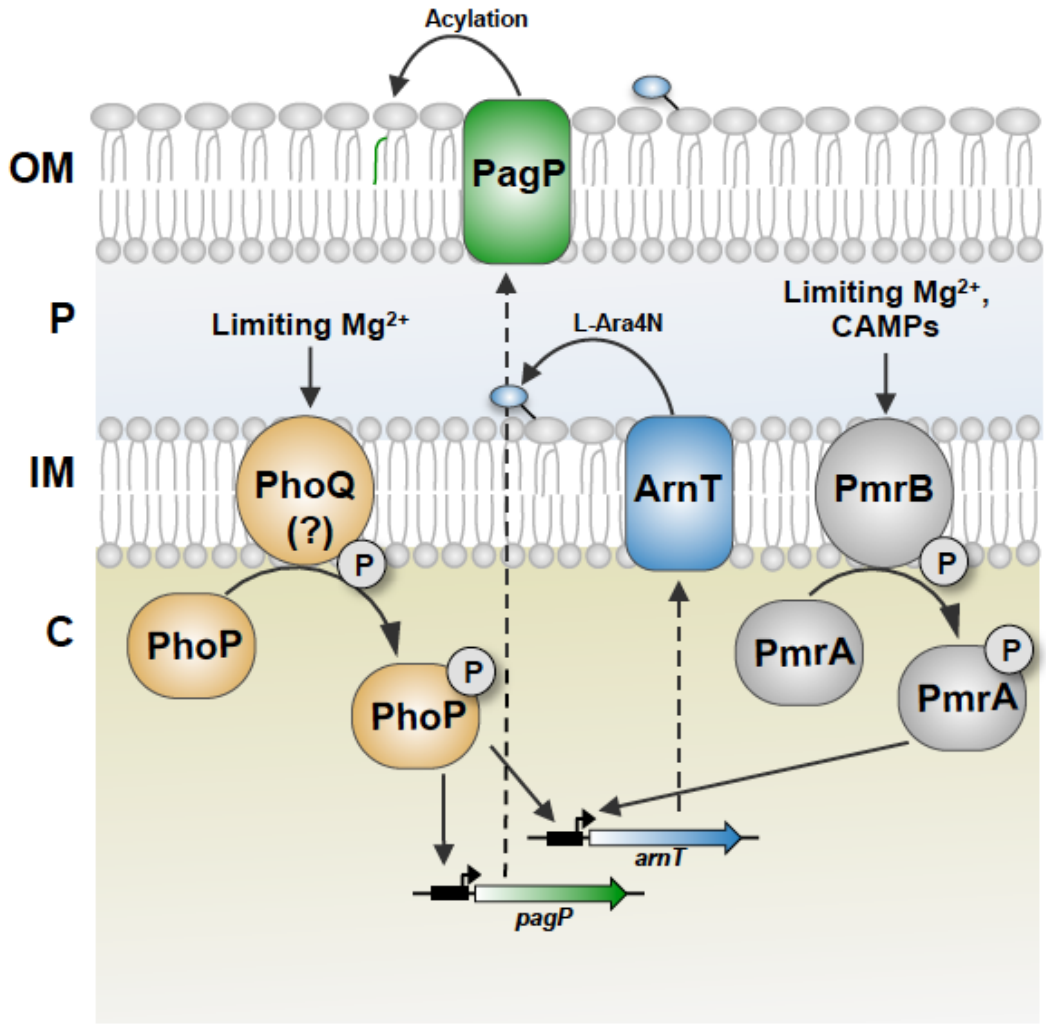

Figure 1.5: Transcriptional regulation of *P. aerugionosa* lipid A modifications by the PhoPQ and PmrAB two-component systems.

The *P. aeruginosa* PhoPQ two-component system, which responds to limiting Mg^{2+}, activates transcription of the lipid A modification genes *pagP* and *arnT*. Transcription of *arnT* is also activated by the PmrAB system independently of PhoPQ in response to limiting Mg^{2+} as well as various CAMPs, including polymyxin.

Aside from PhoPQ and PmrAB, three additional two-component systems are involved in lipid A modification and polymyxin resistance in *P. aeruginosa* (Fig. 1.6). The first two of these systems, ParRS and CprRS, both activate *arnT* transcription in response to various CAMPs (69, 70). Microarray-based transcriptional analysis also indicates that ParRS induces transcription of *pagL*, although this has not yet been experimentally confirmed (70). The third system, ColRS, remains largely unstudied in *P. aeruginosa* but plays a role in heavy metal tolerance and overall membrane stability in *P. putida* and *P. fluorescens* (71–73). A recent study investigated the role of CprRS and ColRS in *P. aeruginosa* polymyxin resistance. This report revealed that these systems can promote polymyxin resistance in a highly resistant Δ*phoQ* strain and also suggested that additional unknown factors contribute to polymyxin resistance (54). Our laboratory has now begun to characterize the role of the ColRS system in lipid A modification. In *P. aeruginosa,* this system responds to Zn^{2+} and activates the transcription of *eptA*, which adds pEtN groups to lipid A that previously had not been detected in this organism. Simultaneously, ColR represses *arnT* transcription in a Zn^{2+}-dependent manner, exerting strict control over which amine-containing modification group will be added to the lipid A (Fig. 1.6)(48). Given the complexities of modification systems in *P. aeruginosa,* continued study of lipid A modification, its regulation, and its role in antimicrobial resistance are necessary.

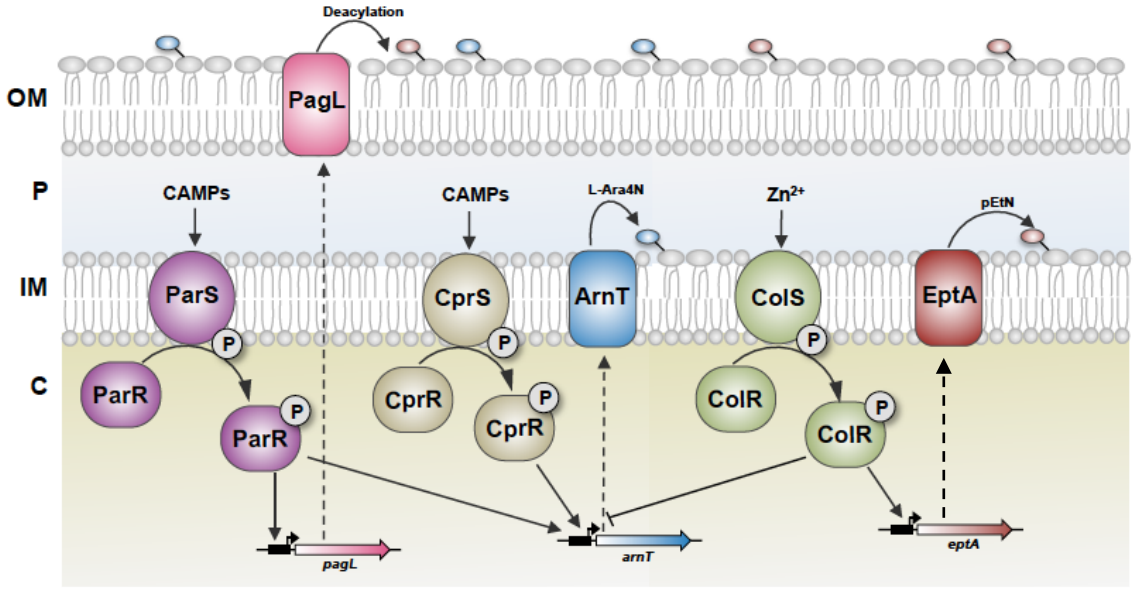

Figure 1.6: Transcriptional regulation of *P. aeruginosa* lipid A modifications by the ParRS, CprRS, and ColRS two-component systems.

The *P. aeruginosa* ParRS and CprRS two-component system both respond to CAMPs and induce transcription of *arnT*. In addition to activating *arnT* transcription, the ParRS system also acts to repress transcription of the lipid A deacylase, *pagL*. The *P. aeruginosa* ColRS system senses Zn^{2+}, and activated ColR then induces transcription of the pEtN transferase *eptA* while repressing *arnT* transcription.

1.3.2 Post-transcriptional regulation

In addition to the complex systems in place to regulate the transcription of lipid A modification genes, control over the activity of these modification enzymes can also occur at the post-translational level. One example is negative regulation of the lipid A kinase, LpxT, upon activation of the PmrAB system. If an *S. enterica* or *E. coli* cell is grown in the presence of excess Fe^{3+}, iron is initially taken up by the cell. Upon activation of PmrAB, however, transcription of the short peptide PmrR is induced. PmrR binds to and prevents LpxT from transferring an additional phosphate group to lipid A (74). The very condition that leads to inhibition of LpxT activity, however, induces transcription of *eptA* (42, 58). Other conditions that either directly or indirectly induce *eptA* transcription, including mild acid pH and limiting Mg^{2+}, also inhibit LpxT activity (42). The 1-phosphate group, sole site of phosphorylation by LpxT and primary site of pEtN addition, is now open to modification by EptA (42). The amine-containing pEtN moiety reduces the overall negative charge of the outer membrane, helping to prevent uptake of Fe^{3+} (74). Similarly, LpxT activity in *P. aeruginosa* is inhibited when Mg^{2+} is limiting, a condition that simultaneously activates *arnT* transcription (31). As in *S. enterica* and *E. coli*, this inhibition of LpxT activity occurs post-transcriptionally (31). The regulation of the outer membrane modifications in response to a changing environment thus appears to be a common phenomenon among different bacterial species.

Modification enzymes acting on the lipid A acyl chains can also be controlled post-translationally. While PagP transcription can be induced by the PhoPQ system when Mg^{2+} is limiting, its activity is also increased under conditions that perturb the outer membrane such as EDTA treatment (75). EDTA causes translocation of phospholipids from the inner leaflet of the outer membrane to the outer leaflet. These phospholipids migrate and find themselves adjacent to the PagP active site, which then cleaves the phospholipid a modifies lipid A in the process (75). Temperature can be another regulatory factor for lipid A modification enzymes as PagL in *P. aeruginosa* shows reduced activity at low growth temperatures (25, 76). It is possible that still uninvestigated mechanisms of post-translational control exist, thus continued study of lipid A modification and regulation in *P. aeruginosa* and all Gram-negatives is extremely important.

Chapter 2: Characterization of *Pseudomonas aeruginosa* LpxT reveals dual positional lipid A kinase activity and coordinated control of outer membrane modification[1]

2.1 INTRODUCTION

Structural modification of lipid A is a common adaptive response to environmental flux, and is often the downstream result of two-component system activation by specific external stimuli (1, 2). For example, transcription of *arnT* in *P. aeruginosa* is primarily induced by the two-component systems PhoPQ and PmrAB, although recent studies have reported the involvement of additional systems (54, 67). Both of these systems respond to limiting Mg^{2+}, while PmrAB can also sense subinhibitory concentrations of CAMPs (67, 68). In *E. coli* and *S. enterica,* EptA competes with LpxT for modification at the 1-phosphate group (42). LpxT activity as a lipid A kinase is inhibited in response to conditions that induce *eptA* expression via the two-component system PmrA-PmrB, such as limiting magnesium or high iron (42, 58, 77). Activation of PmrA induces transcription of a small regulatory peptide, PmrR, which interacts with LpxT to inhibit its activity (74). This leaves the lipid A 1-phosphate group open to pEtN addition by EptA. In this way, the bacterial cell can precisely orchestrate and permit the most beneficial modifications in response to the surrounding environment (42, 74).

[1] Large portions of this chapter have been previously published (copyright by John Wiley and Sons, re-used with permission). **Nowicki EM, O'Brien JP, Brodbelt JS, Trent MS**. 2014. Characterization of *P seudomonas aeruginosa* LpxT reveals dual positional lipid A kinase activity and co-ordinated control of outer membrane modification: Identification of *P. aeruginosa LpxT*. Molecular Microbiology **94**:728-741. (E.M.N. designed and performed experiments and wrote the manuscript under the supervision of M.S.T.; J.P.O. designed and performed the ESI and UVPD-MS experiments under the supervision of J.S.B.).

While LpxT has only been characterized in *E. coli* and *S. enterica, tris*-phosphorylated lipid A species have previously been reported in other Gram-negatives including *Neisseria meningitidis* and *Yersinia pestis* (78–80). Despite the implication that *lpxT* orthologs are widespread, there remains a lack of studies examining the biochemistry and regulation of these orthologs. Work in *E. coli* and *S. enterica* has revealed the importance of *lpxT* regulation to promote the addition of positively charged moieties to lipid A; whether such coordinated regulation exists in other Gram-negatives is currently unknown (42, 74). We therefore set out to determine the existence of an LpxT ortholog in *P. aeruginosa* and, if present, how this enzyme is regulated.

Here we report the identification and characterization of a functional LpxT ortholog in *P. aeruginosa.* We show that $LpxT_{Pa}$ activity is distinct from that of $LpxT_{Ec}$ in that it can add not only to the 1-phosphate, but the 4'-position as well. We also describe the first reported example of a lipid A 1-triphosphate species. Further, we demonstrate that while phosphate group addition to *P. aeruginosa* lipid A readily occurs when Mg^{2+} concentration is high, $LpxT_{Pa}$ activity is inhibited when Mg^{2+} is limiting, and instead L-Ara4N modification occurs via the enzyme ArnT. Deletion of *arnT,* however, results in partial restoration of $LpxT_{Pa}$ activity, suggesting coordinated regulation of modification at the lipid A phosphate groups. Further investigation of $LpxT_{Pa}$ and how it is regulated will allow better understanding of lipid A modification machinery in this important opportunistic pathogen.

2.2 RESULTS

2.2.1 *P. aeruginosa* lipid A is modified with an additional phosphate group

Reports of *tris*-phosphorylated lipid A in several different organisms suggest that *lpxT*-mediated phosphorylation of lipid A is widespread across Gram-negatives (78–80). We began our search for an *lpxT* ortholog in *P. aeruginosa* by performing a BLAST (NCBI) bioinformatics analysis to identify highly similar proteins to K-12 *E. coli lpxT* ($lpxT_{Ec}$). A single candidate with significant similarity, PA14_68620 was identified, having 30% sequence identity and an e-value of $1e10^{-18}$. This gene is conserved among members of the *Pseudomonas* genus, according to the Pseudomonas Genome Database (81).

The existence of this ortholog (herein referred to as $lpxT_{Pa}$) prompted us to determine whether *P. aeruginosa* lipid A is modified with an additional phosphate group. Lipid A was isolated from *P. aeruginosa* grown in MOPS minimal medium supplemented with 2mM $MgSO_4$ and analyzed by MALDI-TOF mass spectrometry (MS). A $MgSO_4$ concentration of 2mM was used, as this concentration of divalent cations has been reported to occur in the human body (70). Lipid A *tris*-phosphate was clearly detected for both the predominant, penta-acylated species and the less-abundant hexa-acylated species, generating major molecular ions at *m/z* 1525.6 and 1695.7, respectively

(predicted $[M-H]^-$ at m/z 1525.8 and 1696.0) (Fig. 2.1A and 2.1B). This result confirms that *P. aeruginosa* lipid A can be *tris*-phosphorylated.

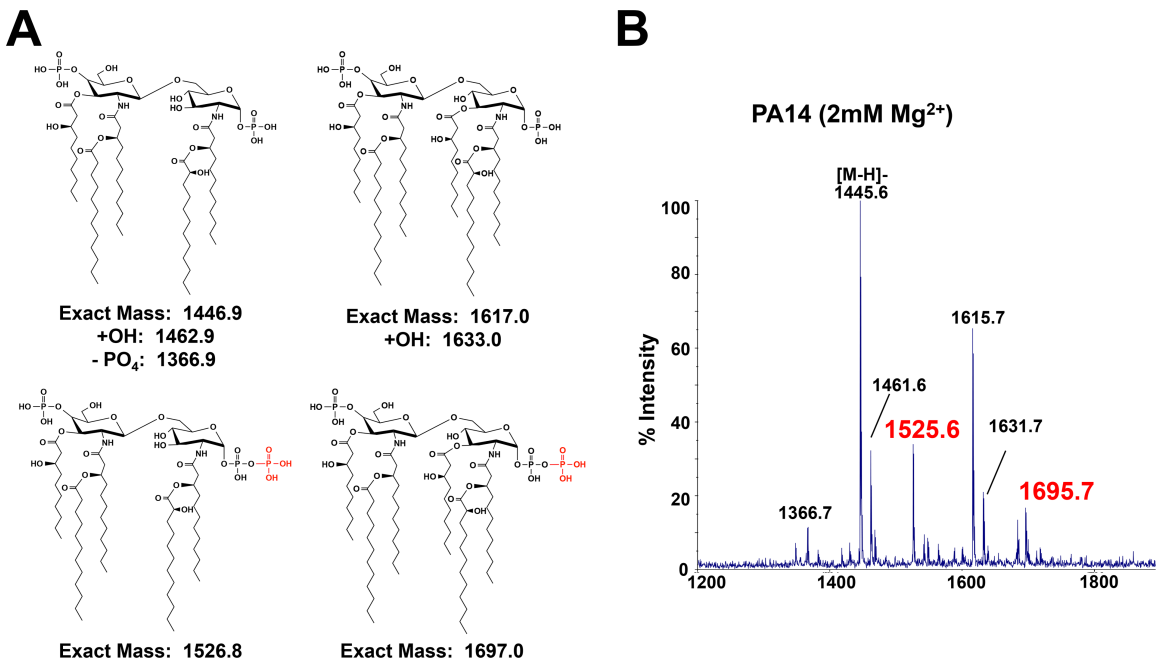

Figure 2.1: *P. aeruginosa* lipid A is modified with an additional phosphate group.

A) Structures of the predominant penta- and hexa-acylated lipid A species and their exact masses are shown, with the corresponding phosphate-modified species shown below. Phosphate group addition is highlighted in red. B) MALDI-TOF MS analysis of the 480mM ammonium acetate lipid A eluate isolated from *P. aeruginosa* grown in 1 L MOPS minimal medium supplemented with 2 mM $MgSO_4$ shows penta- and hexa-acylated lipid A species (m/z at 1445.6 and 1615.7, respectively) as well as penta- and hexa-acylated lipid A *tris*-phosphate (m/z at 1525.6 and 1695.7, respectively; red).

2.2.2 Identification of a functional LpxT ortholog in *P. aeruginosa*

Given that *P. aeruginosa* lipid A can be modified with an additional phosphate group, the next goal was to determine whether $lpxT_{Pa}$ (PA14_68620) is the enzyme responsible for this modification. $lpxT_{Pa}$ was expressed *in trans* in an *E. coli lpxT* mutant (W3110Δ$lpxT_{Ec}$), and $^{32}P_i$-labelled lipid A from this strain was isolated and separated by TLC. The migration of *E. coli* lipid A in our TLC solvent system has been well-characterized, and consists of two major species: approximately two-thirds *bis*-phosphorylated lipid A, and one-third *tris*-phosphorylated lipid A (lipid A 1-diphosphate) due to $LpxT_{Ec}$ activity (Fig. 2.2A, lane 1) (42). Deletion of $lpxT_{Ec}$ results in a single, *bis*-phosphorylated lipid A species (Fig. 2.2A, lane 2). Both $lpxT_{Ec}$ and $lpxT_{Pa}$ were able to complement the $lpxT_{Ec}$ mutant, as shown by the restoration of the lipid A *tris*-phosphate species (Fig. 2.2A, lanes 3 and 4). $LpxT_{Pa}$ activity was confirmed by MALDI-TOF mass spectrometry, in which $lpxT_{Pa}$-dependent phosphorylation of lipid A corresponds to the peak at m/z 1876.3 (predicted $[M-H]^-$ at m/z 1876.2) (Fig. 2.2B & 2.2C).

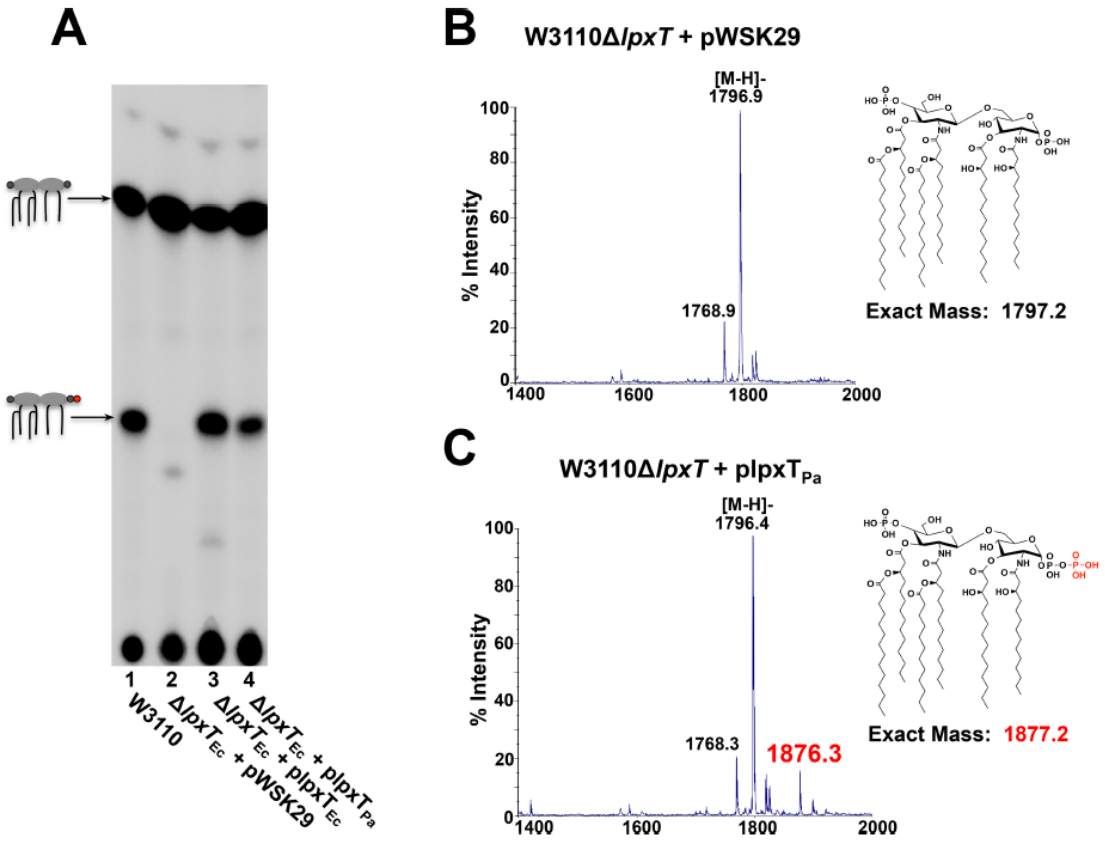

Figure 2.2: Heterologous expression of the *P. aeruginosa lpxT* ortholog restores *tris*-phosphorylated lipid A species in an *E. coli* W3110Δ*lpxT* mutant.

A) Cells were grown in LB medium. Major ^{32}P-labelled lipid A species are indicated with a cartoon of the corresponding structure; colors of groups added to the lipid A molecule correspond to colors used in Fig. 1. Wild-type *E. coli* W3110 lipid A is isolated as two major species, two-thirds *bis*-phosphorylated lipid A with phosphates at the 1 and 4' positions, and one-third *tris*-phosphorylated lipid A, or lipid A 1-diphosphate (Lane 1). TLC separation of lipid A shows that *lpxT*$_{Pa}$, like *lpxT*$_{Ec}$, can modifiy *E. coli* lipid A with a phosphate group (lanes 3 and 4). B & C) MALDI-TOF analysis of lipid A isolated from W3110Δ*lpxT* + empty vector (pWSK29) (B) and W3110Δ*lpxT* + p*lpxT*$_{Pa}$ (C) demonstrates that *lpxT*$_{Pa}$ can modify *E. coli* lipid A with an additional phosphate group (m/z at 1876.3, red).

2.2.3 An lpxT$_{Pa}$ deletion mutant cannot phosphorylate lipid A

The necessity of *lpxT*$_{Pa}$ for phosphate modification of *P. aeruginosa* lipid A was next examined by generating a chromosomal deletion mutant of *lpxT*$_{Pa}$. Lipid A from $^{32}P_i$-labelled wild-type, PA14Δ*lpxT*$_{Pa}$, and complemented mutant strains was isolated from cells grown in MOPS minimal medium supplemented with 2 mM $MgSO_4$ and separated by TLC. Deletion of *lpxT*$_{Pa}$ resulted in loss of a major lipid A species predicted to be *tris*-phosphorylated lipid A (Fig. 2.3A, lane 3) that was restored upon complementation with *lpxT*$_{Pa}$ (Fig. 2.3A, lane 4). Samples isolated from *P. aeruginosa* grown in rich medium (LB) also showed LpxT$_{Pa}$-dependent phosphorylation of lipid A (Fig. 2.4). The lack of a lipid A *tris*-phosphate species in the *lpxT*$_{Pa}$ deletion mutant was confirmed by MALDI-TOF and ESI-MS (Figs. 2.3B and 2.5). In the MALDI-TOF spectrum of the complemented mutant strain, however, additional peaks corresponding to *tris*-phosphorylated lipid A species were easily detected (Fig. 2.3C). *lpxT*$_{Pa}$ is thus required for phosphate modification of *P. aerguinosa* lipid A.

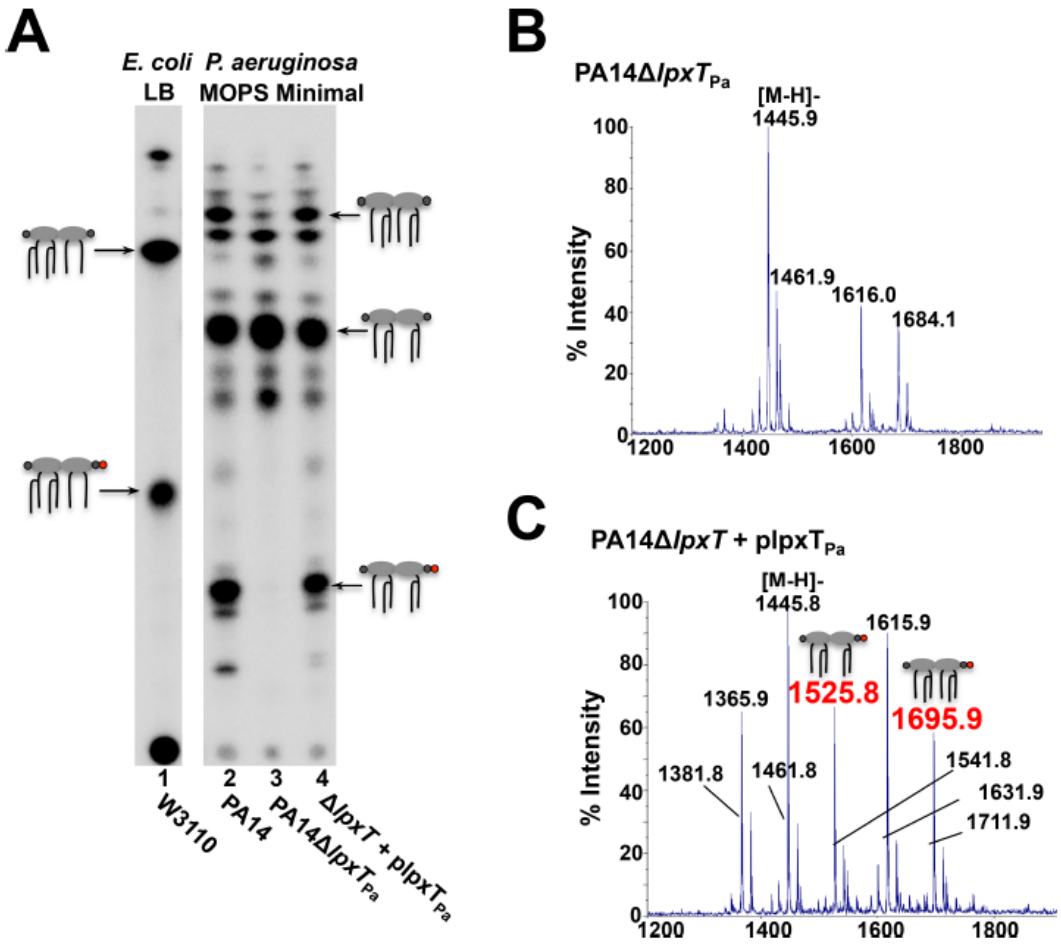

Figure 2.3: *lpxT*$_{Pa}$ is necessary for phosphorylation of *P. aeruginosa* lipid A.

A) *E. coli* was grown in LB, *P. aeruginosa* was grown in MOPS minimal medium supplemented with 2 mM $MgSO_4$. Minor lipid A species below major *P. aeruginosa* species correspond to hydroxylated forms of lipid A due to LpxO activity. TLC separation of lipid A shows absence of *tris*-phosphorylated lipid A when *lpxT*$_{Pa}$ is deleted (lane 3). Upon complementation with p*lpxT*$_{Pa}$, lipid A *tris*-phosphate is restored (lane 4). B) MALDI-TOF MS analysis of the 480mM ammonium acetate lipid A eluate isolated from PA14Δ*lpxT*$_{Pa}$ grown in 1 L MOPS minimal medium supplemented with 2 mM $MgSO_4$ shows no peaks corresponding to *tris*-phosphorylated lipid A. C) MALDI-TOF MS analysis of the 480mM elution fraction of lipid A reveals that when *lpxT*$_{Pa}$ is expressed *in trans* in the PA14Δ*lpxT*$_{Pa}$ mutant, phosphorylation of both penta- and hexa-acylated lipid A is restored (m/z at 1525.8 and 1695.8, respectively; red).

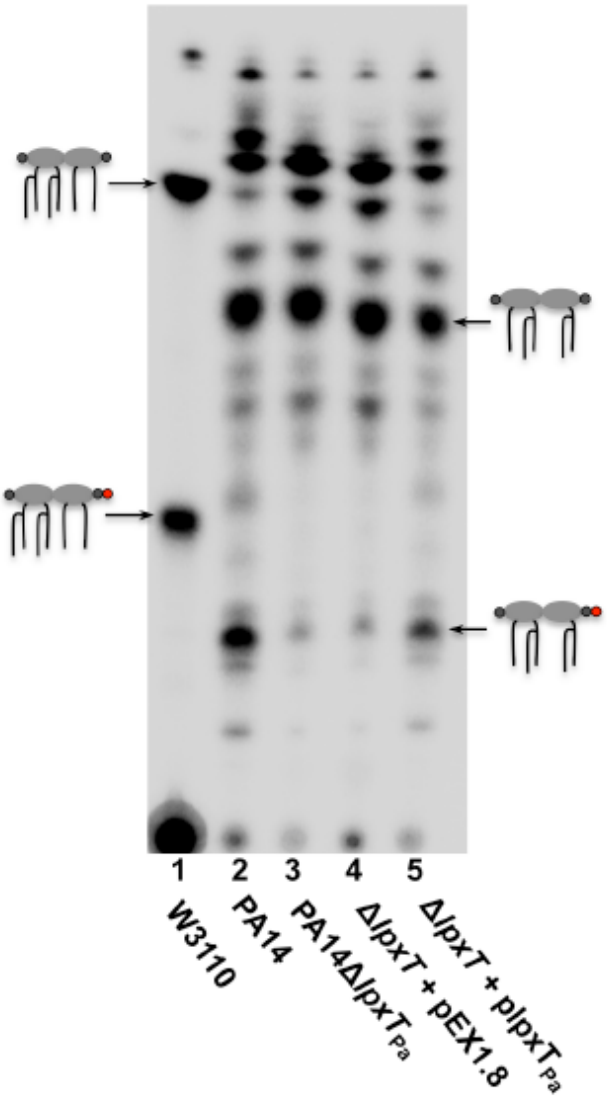

Figure 2.4: $LpxT_{Pa}$ is active in LB medium.

E.coli strain W3110 and *P. aeruginosa* were grown in LB broth. *Tris*-phosphorylated *P. aeruginosa* lipid A is detected when cells are grown in LB medium at 37°C (lane 2). When $lpxT_{Pa}$ is deleted, the lipid A *tris*-phosphate species is absent from the TLC plate (lane 3). When this strain is complemented *in trans* with p$lpxT_{Pa}$, the lipid A *tris*-phosphate species is restored (lane 4).

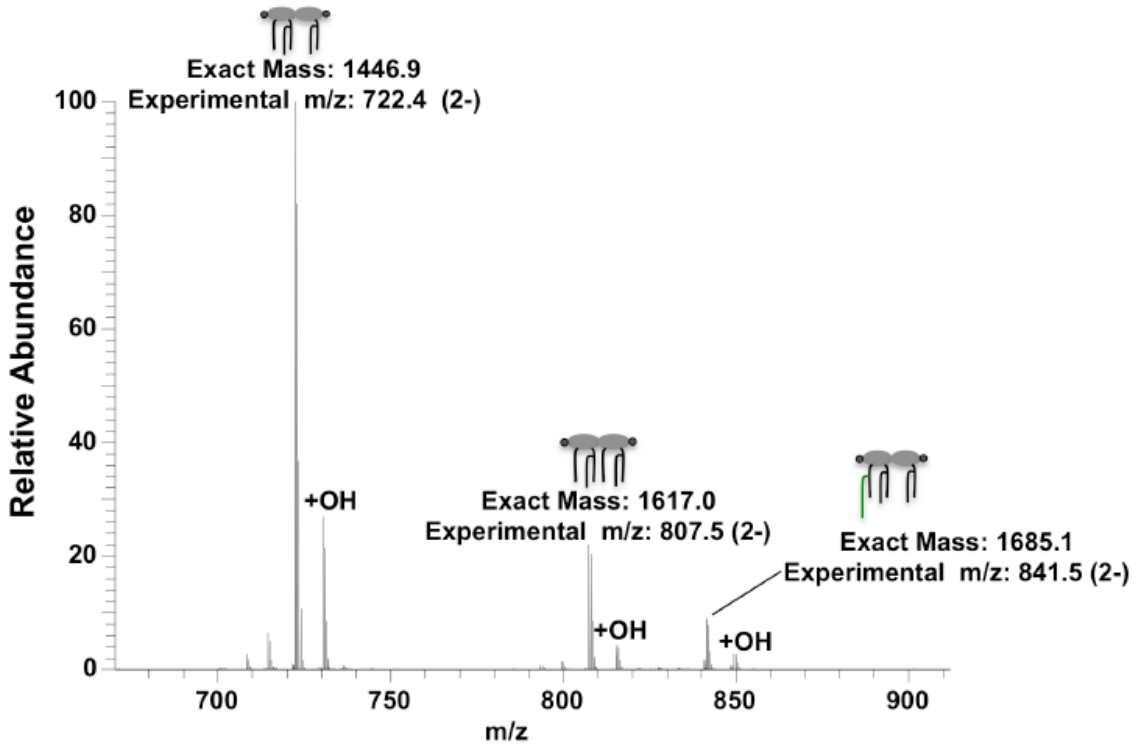

Figure 2.5: Negative ion mode ESI mass spectrum of *P. aeruginosa* PA14ΔlpxT.

The mass spectrum showed the presence of wild type penta-acyl lipid A [Exact mass = 1446.9 Da], wild type lipid A [Exact mass 1617.0 Da], and a PagP hexa-acylated lipid A [Exact mass 1685.1 Da]. There was no evidence of lipid A with an additional phosphate group.

2.2.4 *P. aeruginosa* and *E. coli* LpxT have divergent activity

Previous analysis of *tris*-phosphorylated *E. coli* lipid A has detected phosphate group addition exclusively at the 1-position (30, 82). The next approach was to determine whether $LpxT_{Pa}$ has a similar site-specificity to that of $LpxT_{Ec}$. Lipid A was isolated from *P. aeruginosa* grown in MOPS minimal medium supplemented with 2mM $MgSO_4$ and analyzed first by ESI-MS (Fig. 2.6A) and subsequently by ultraviolet photodissociation mass spectrometry (UVPD-MS) (Fig. 2.6C), resulting in ions from multiple cleavages of the lipid A parent molecules (selected precursor m/z 762.4). UVPD-MS analysis showed that unlike in *E. coli*, phosphate modification of *P. aeruginosa* lipid A can occur both at the 1-phosphate and the 4'-phosphate groups (Figs. 2.6B and 2.7A & B). Cleavages generating molecular ions at m/z 718.34, 734.33, and 806.49 allowed identification of the 1-diphosphate species, while ions at m/z 636.45, 654.36, 871.46, and 886.45 provided strong evidence for a 4'-diphosphate species (Figs. 2.6C and 2.7A & B). Interestingly, a low abundance of lipid A 1-triphosphate was also identified (Figs. 2.6B and 2.7C). UVPD-MS resulted in fragment ions at m/z 256.90 and 814.30 thus confirming the identity of the 1-triphosphate species (Figs. 2.6C and 2.7C). This unique species is especially surprising given that in addition to the 1-position being modified with two additional phosphate groups, the 4' phosphate of the glucosamine backbone is absent. This not only demonstrates a previously uncharacterized activity of LpxT, but also suggests the existence of a lipid A phosphatase in *P. aeruginosa,* as such an enzyme is necessary to cleave the lipid A 4' phosphate (1, 2).

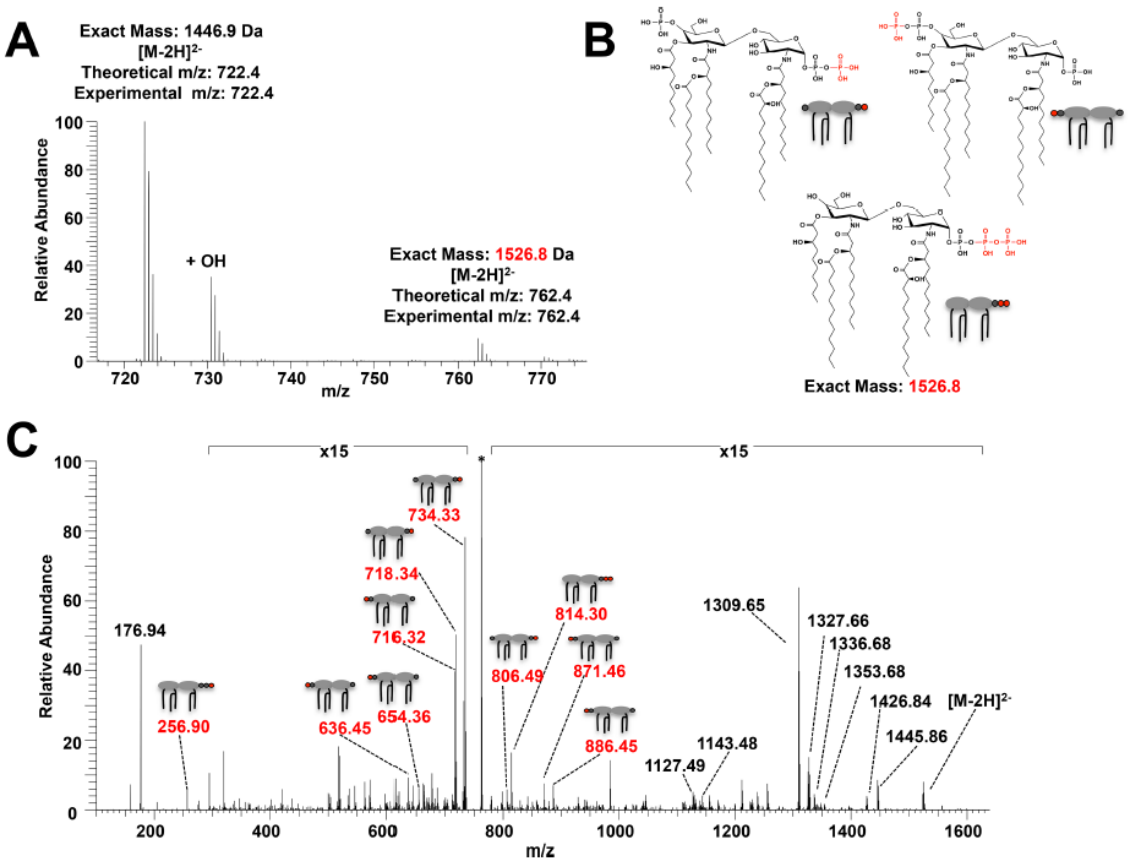

Figure 2.6: $LpxT_{Pa}$-dependent phosphorylation of *P. aeruginosa* lipid A results in multiple *tris*-phosphorylated species.

A) Negative ion mode ESI mass spectrum of PA14 *P. aeruginosa* revealing a mixture of $[M-2H]^{2-}$ lipid A corresponding to wild type penta-acylated lipid A [Exact mass = 1446.9 Da] and a species corresponding to an addition of one phosphate group [Exact Mass = 1526.8 Da]. B) A mixture of three distinct lipid A species (1-pyrophosphate, 4′-pyrophosphate, and 1-triphosphate species) were identified through the C) UVPD mass spectrum of the $[M-2H]^{2-}$ *m/z* 762.4 from (A). Key fragment ions corresponding to each of the different lipid A types are highlighted in red with the corresponding precursor structure assignment while the remaining fragment ions revealed the lipid A acylation pattern. Fragmentation cleavage maps for the identified species are shown in Fig. 2.7 and the precursor ion is marked with an asterisk.

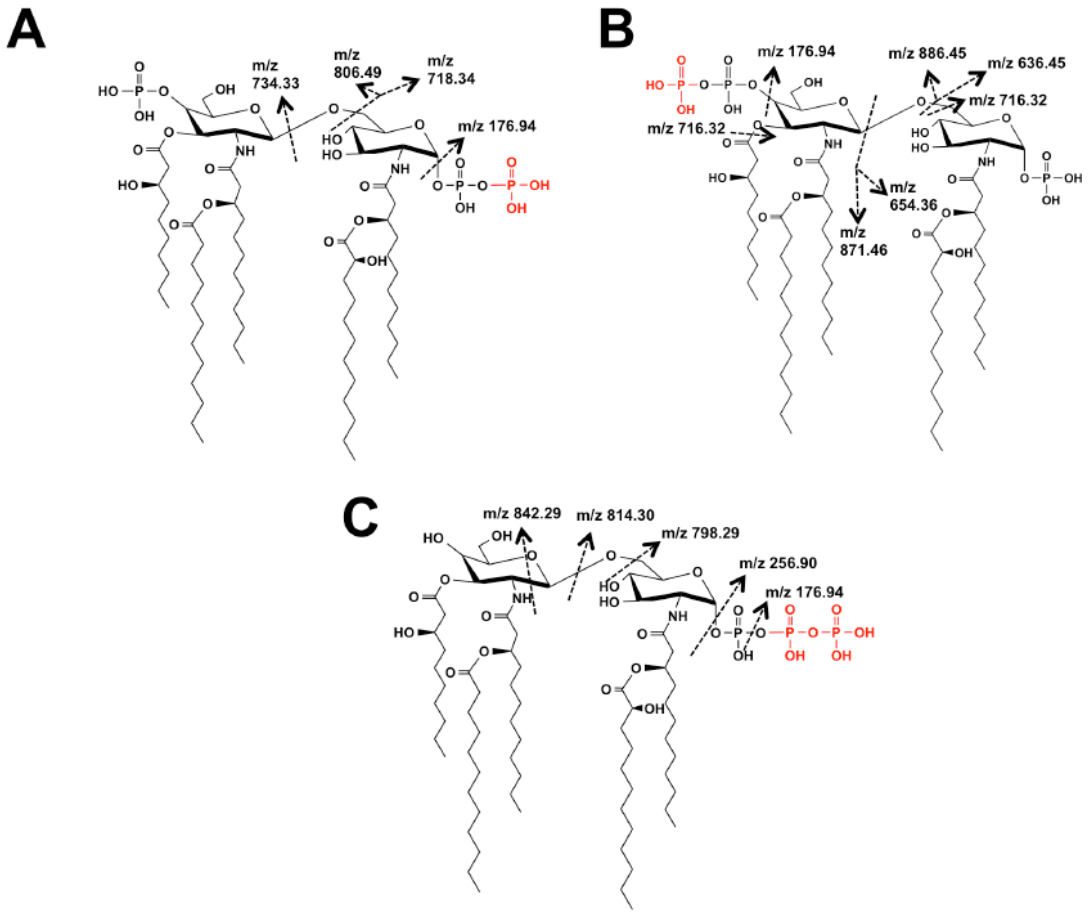

Figure 2.7: Fragment ion cleavage maps for the (A) 1-pyrophosphate species, (B) 4′-pyrophosphate species, and (C) 1-triphosphate species upon UVPD of $[M-2H]^{2-}$ *m/z* 762.4 (from Fig. 2.6A). The corresponding UVPD mass spectrum is shown in Figure 2.6C.

2.2.5 $LpxT_{Pa}$ activity is modulated by Mg^{2+}

Lipid A modification with positively charged L-Ara4N and pEtN groups by the enzymes ArnT and EptA, respectively, improves bacterial survival in deleterious conditions such as limiting magnesium and exposure to cationic antimicrobial peptides(83)(1, 2). Modulation of LpxT activity permits pEtN modification at the 1-phosphate group by EptA, without competition from LpxT (42, 74). The *P. aeruginosa* L-Ara4N transferase can modify both phosphate groups of lipid A (34) and according to our findings the same can be said for $LpxT_{Pa}$. Therefore, we speculated that $LpxT_{Pa}$ activity might be inhibited under conditions that induce *arnT* transcription to prevent competition between phosphate group and L-Ara4N addition. To test this possibility, lipid A was isolated from $^{32}P_i$-labelled *P. aeruginosa* cells grown in MOPS minimal medium supplemented with either 2 or 0.02 mM $MgSO_4$ (high or low Mg^{2+}), the latter of which has been previously shown to induce *arnT* transcription via PhoPQ and PmrAB (67, 68). Growth in low Mg^{2+} resulted in absence of the *tris*-phosphorylated lipid A species and the appearance of L-Ara4N and palmitate-modified lipid A species due to ArnT and PagP activity, respectively (Fig. 2.8A, lane 3). Absence of *tris*-phosphorylated lipid A was confirmed by MALDI-TOF MS (Fig. 2.8B). Double L-Ara4N-modified species were also detected in our MS analysis of the wash fraction of this lipid A sample (Fig. 2.9). These results suggest that limiting Mg^{2+} during growth results in inhibition of $LpxT_{Pa}$ activity, either by influencing $lpxT_{Pa}$ transcription or enzyme activity.

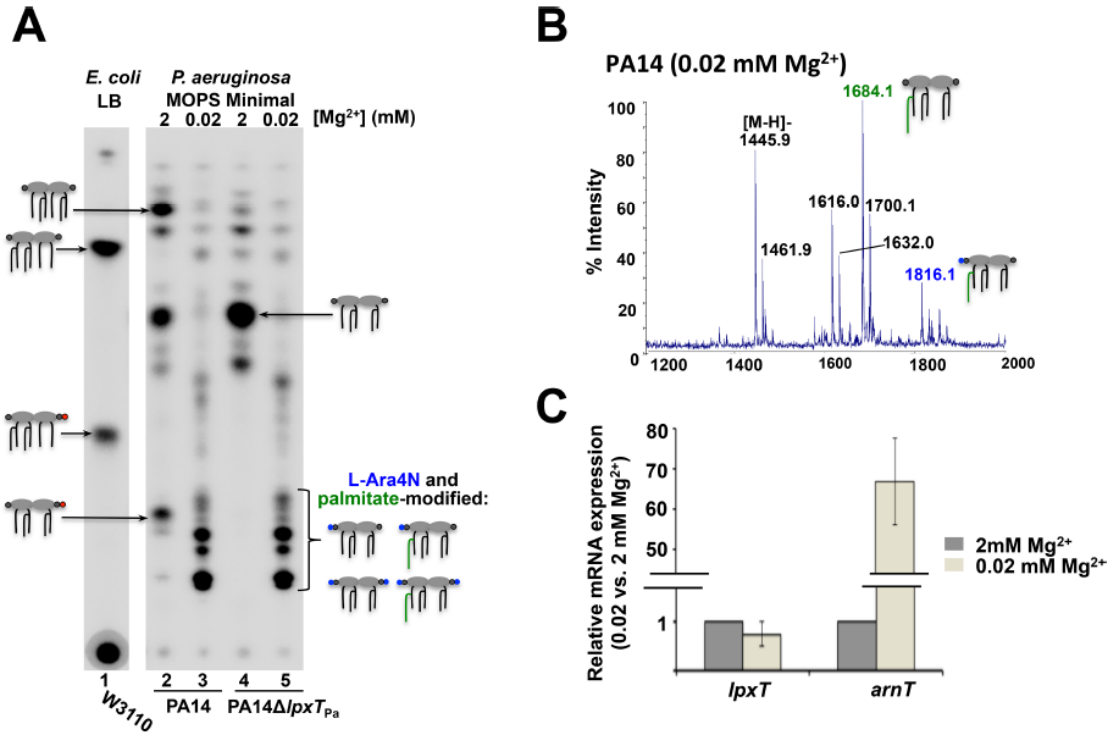

Figure 2.8: $LpxT_{Pa}$ activity is modulated by magnesium.

A) *E. coli* was grown in LB, *P. aeruginosa* was grown in MOPS minimal medium supplemented with 2 or 0.02 mM $MgSO_4$, as indicated. When grown in low magnesium, lipid A *tris*-phosphate was not detected by TLC separation (lane 3). Instead, L-Ara4N and palmitate-modified species were observed for both PA14 and PA14Δ*lpxT* grown in low magnesium (lanes 3 and 5). B) MALDI-TOF MS analysis of the 480mM ammonium acetate lipid A eluate shows the absence of *tris*-phosphorylated species in PA14 grown in MOPS minimal medium supplemented with 0.02 mM $MgSO_4$. Palmitoylation of the penta-acylated species (m/z at 1684.1) is indicated in green. C) Relative gene expression of $lpxT_{Pa}$ and *arnT* when grown in MOPS minimal medium supplemented with 0.02 mM $MgSO_4$ compared to 2 mM $MgSO_4$ shows that while $lpxT_{Pa}$ transcription does not significantly change (<1.5-fold difference) with respect to magnesium concentration, *arnT* transcription significantly increases in 0.02 mM $MgSO_4$.

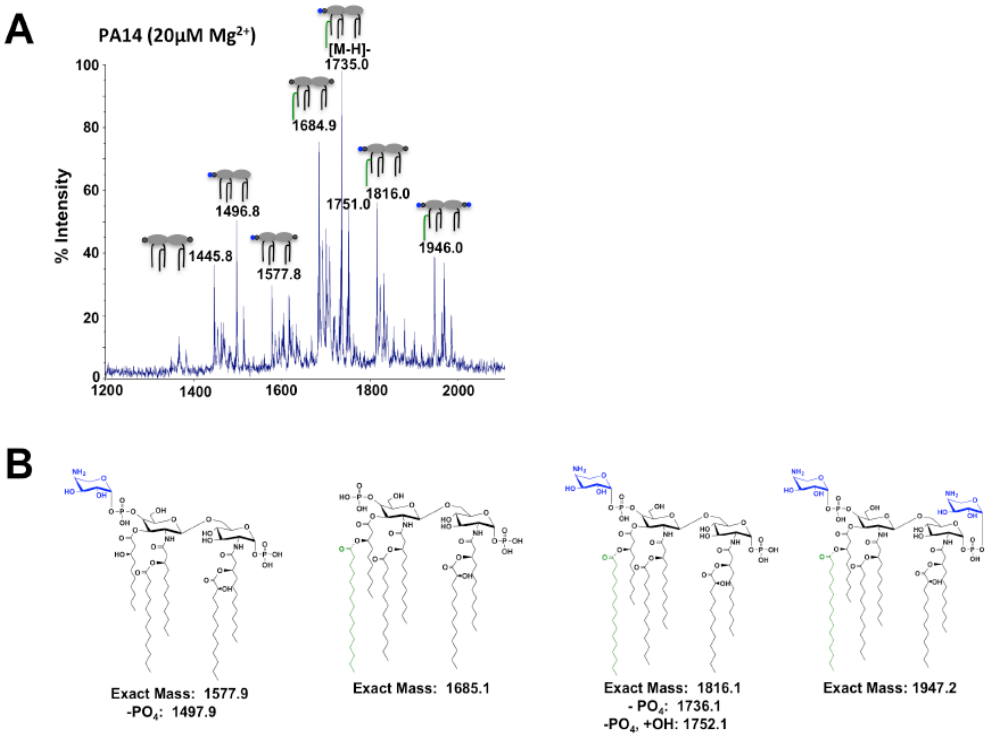

Figure 2.9: Limiting magnesium induces *arnT* and *pagP* transcription, resulting in L-Ara4N and palmitate modification.

A) MALDI-TOF MS of the wash fraction of lipid A isolated from *P. aeruginosa* grown in MOPS minimal supplemented with 0.02 mM $MgSO_4$ shows abundant L-Ara4N, double L-Ara4N (on either lipid A phosphate group), and palmitate-modified lipid A species. B) Structures of lipid A species grown in limiting magnesium depicting L-Ara4N and palmitate modifications

It was then investigated whether Mg^{2+}-dependent modulation of $LpxT_{Pa}$ occurs at the transcriptional level. Quantitative PCR was performed to compare the level *of* $lpxT_{Pa}$ expression in high or low Mg^{2+}, using the housekeeping gene *clpX* as an internal control (84). The transcription of *arnT* was also analyzed under the same conditions as a positive control for Mg^{2+} regulation. While *arnT* transcription is strongly induced when Mg^{2+} is limiting, the transcription of $lpxT_{Pa}$ does not significantly change (<1.5-fold difference) (Fig. 2.8C). Mg^{2+}-dependent modulation of $LpxT_{Pa}$ activity is thus independent of $lpxT_{Pa}$ transcription, as is the case in *E. coli* and *S. enterica* (42, 74).

2.2.6 $LpxT_{Pa}$ activity in low Mg^{2+} is partially restored in the PA14ΔarnT mutant

In *E. coli* and *S. enterica,* although LpxT-dependent lipid A phosphorylation at the 1-phosphate group occurs under normal laboratory growth conditions, its activity is inhibited when *eptA* transcription is activated by environmental stimuli (42, 74). If *lpxT* is deleted, however, a small amount of pEtN-modified lipid A is detected even under conditions that do not normally induce *eptA* transcription (42). Given that ArnT can add L-Ara4N to both phosphate groups of lipid A (Fig. 2.9) (34), we questioned whether a similar mechanism occurs in *P. aeruginosa* to control phosphate group and L-Ara4N addition thereby reducing their competition. Since deletion of $lpxT_{Pa}$ does not result in detectable L-Ara4N-modified lipid A when Mg^{2+} concentration is high (Fig. 2.3), we wondered if deletion of *arnT* would result in $LpxT_{Pa}$ activity in low Mg^{2+}. Accordingly, $^{32}P_i$-labelled lipid A was isolated from PA14Δ*arnT* grown in MOPS minimal medium

supplemented with high or low Mg^{2+} and separated by TLC (Fig. 2.10). When *arnT* is absent from the genome, the lipid A *tris*-phosphate species was partially restored in cells grown in low Mg^{2+} (Fig. 2.10A, lane 3). Partial restoration of the *tris*-phosphorylated lipid A species in the PA14Δ*arnT* mutant grown in low Mg^{2+} was further confirmed by MALDI-TOF MS (Fig. 2.10C). Together, these results suggest that coordinated control of lipid A modification at the phosphate groups occurs in *P. aeruginosa*.

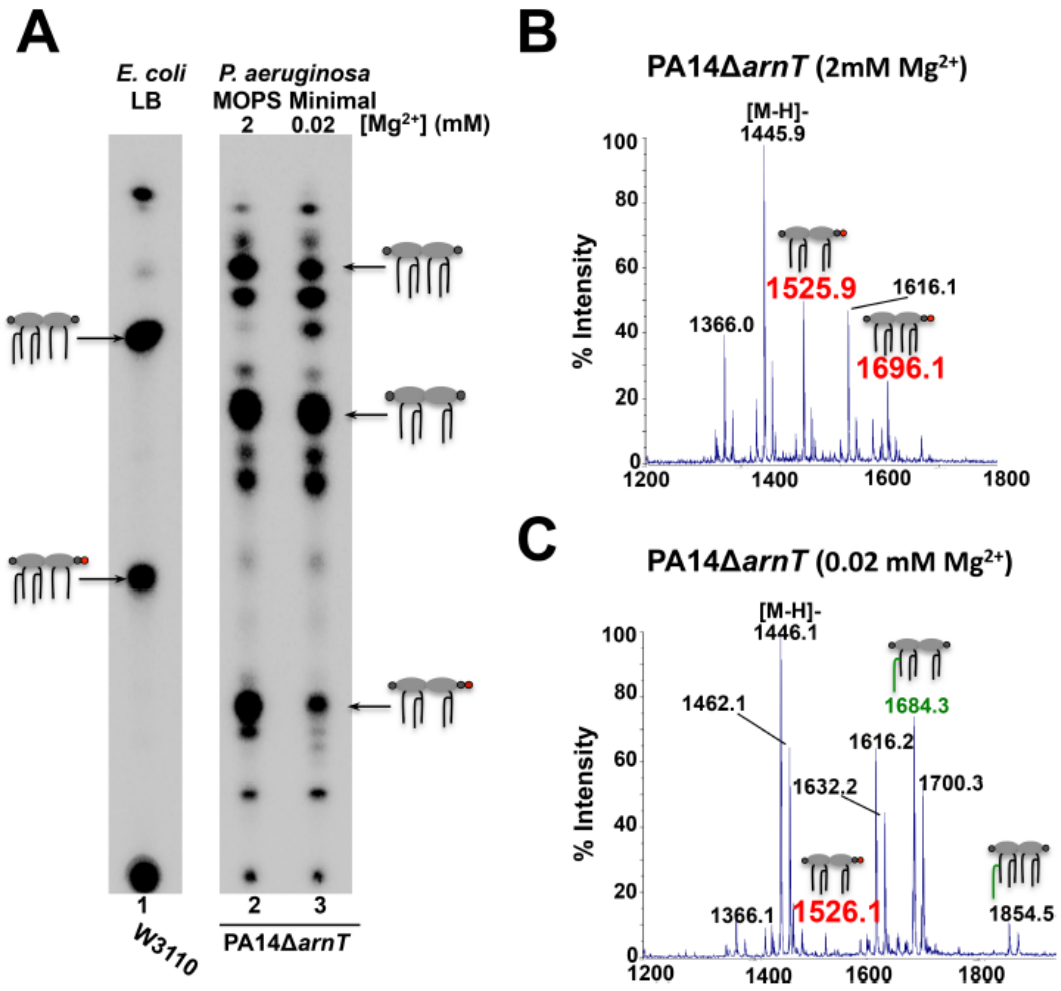

Figure 2.10: $LpxT_{Pa}$ activity in low magnesium is partially restored in the PA14Δ*arnT* mutant.

A) *E. coli* was grown in LB, *P. aeruginosa* was grown in MOPS minimal medium supplemented with 2 or 0.02 mM $MgSO_4$ as indicated. When *arnT* is deleted, a small amount of *tris*-phosphorylated lipid A is detected in lipid A isolated from cells grown in low magnesium (lane 3). B) When PA14Δ*arnT* is grown in MOPS minimal medium supplemented with 2 mM $MgSO_4$, *tris*-phosphorylated lipid A species are readily detected in the 480mM ammonium acetate lipid A eluate fraction (m/z at 1525.8 and 1695.9, respectively; red). C) *Tris*-phosphorylated lipid A species isolated from PA14Δ*arnT* grown in MOPS minimal medium supplemented with 0.02 mM $MgSO_4$ are detected as a minor species (m/z at 1526.1; red).

2.3 DISCUSSION

Modulation of the bacterial outer membrane is crucial for bacterial survival in a changing and potentially deleterious environment. When outer membrane integrity remains uncompromised (for example, when Mg^{2+} is plentiful), phosphate modification of lipid A occurs readily through the action of LpxT (30, 42). However, when Mg^{2+} becomes scarce or cationic antimicrobial peptides are present, modification of lipid A with L-Ara4N, pEtN, or palmitate increase bacterial fitness (1, 2, 67, 68, 85). Environmental stimuli are sensed by two-component regulatory systems that orchestrate outer membrane remodelling by controlling the expression or activity of various lipid A modification enzymes in a coordinated manner (1, 2). For example, phosphorylation of lipid A must be inhibited, while simultaneously promoting the addition of cationic groups at the lipid A phosphates to increase membrane stability (42, 74).

Previous studies have shown the presence of free pyrophosphate groups in *P. aeruginosa* lipid A samples analyzed by mass spectrometry, implicating that lipid A from this organism can be *tris*-phosphorylated (80). We have now identified a *P. aeruginosa* LpxT ortholog that can function on both *E. coli* and *P. aeruginosa* lipid A (Figs. 2.2 and 2.3). As is the case in *E. coli* and *S. enterica*, phosphate group addition occurs under normal laboratory growth conditions in which Mg^{2+} concentration is replete (Figs. 2.1B and 2.4). In all three organisms, limiting Mg^{2+} concentration inhibits LpxT activity while promoting expression of enzymes that add positively charged groups to lipid A. Importantly, there are several critical differences in $LpxT_{Pa}$ activity from that of *E. coli* or *S. enterica*, which are highlighted in Table 2.1. These differences include the dual

positional specificity of $LpxT_{Pa}$ for lipid A modification and competition with ArnT rather than EptA (Table 2.1).

Table 2.1. Summary of LpxT lipid A enzymatic characteristics

Property of LpxT	**Microorganism**	
	E. coli/S. enterica	***P. aeruginosa***
Kinase activity at 1-phosphate group?	Yes, adds one phosphate group	Yes, adds one or two phosphate groups
Kinase activity at 4'-phosphate group?	No	Yes, adds one phosphate group
Conditions known to inhibit activity	Low [Mg^{2+}], mildly acidic pH, excess [Fe^{3+}]	Low [Mg^{2+}]
Mg^{2+}-dependent inhibition: transcriptional or post-transcriptional?	Post-transcriptional	Post-transcriptional
Regulatory system involved in LpxT inhibition	PmrA-PmrB, via the small peptide PmrR	Unknown, no PmrR ortholog present
Competition with other lipid A modification enzymes?	Yes, competes with EptA for modification at 1-phosphate group	Yes, competes with ArnT for modification at both phosphate groups
Other factors related to activity or expression	Basal EptA activity when *lpxT* is deleted	$LpxT_{Pa}$ active in low [Mg^{2+}] when *arnT* is deleted

Previous work in *E. coli* has demonstrated that LpxT is a member of the undecaprenyl-pyrophosphate (Und-PP) phosphatase family of enzymes, which remove a phosphate group from the carrier lipid Und-PP (30). Und-P is a vital molecule required for shuttling polysaccharide intermediates involved in the synthesis of important polymers such as peptidoglycan and O-antigen across the inner membrane (30, 86). By removing a phosphate group from Und-PP, LpxT regenerates Und-P and thus plays a role in the cycle of bacterial cell envelope synthesis (86), while also modifying the lipid A anchor of LPS (30). In *E. coli*, three additional Und-PP phosphatases (YbjG, PgpB, and UppP) are also present that can regenerate Und-P (86, 87). UppP (formerly named BacA) is responsible for the majority of Und-PP dephosphorylation, while the other three account for the remaining activity (86). Like LpxT, PgpB has activity on substrates other than Und-PP; it is one of three enzymes in *E. coli* that functions as a phosphatidylglycerolphosphate phosphatase (87–89). These enzymes dephosphorylate phosphatidylglycerolphosphate, a necessary intermediate in the synthesis of the phospholipid phosphatidylglycerol (88). Although they have not yet been characterized, homologs of the other Und-PP phosphatases exist in *P. aeruginosa* as well.

Characterization of $LpxT_{Pa}$ positional specificity revealed that this enzyme has unique activity in that it can phosphorylate both the 1- and the 4' phosphate groups of lipid A (Figs. 2.6 and 2.9), while LpxT of *E. coli* modifies strictly the 1-position with a single phosphate group (82). We have also demonstrated that deletion of $lpxT_{Pa}$ results in loss of any lipid A *tris*-phosphate species, suggesting that $LpxT_{Pa}$ is both necessary and responsible for phosphorylation of both positions of lipid A (Figs. 2.3A and B; 2.5).

Quite surprisingly, we observed that $LpxT_{Pa}$ adds not only one phosphate group to the 1- or 4'-position of lipid A, but in some cases it can add two phosphate groups at the 1-position, resulting in a minor lipid A 1-triphosphate species (Figs. 2.6 and 2.7). This novel species has never been reported in any microorganism. While it is unknown whether a specific regulatory condition exists that might promote such activity of $LpxT_{Pa}$, under the growth conditions used, this 1-triphosphate lipid A species is in very low abundance compared to 1- or 4'-diphosphate lipid A species.

Another unexpected feature of the 1-triphosphate species is its lack of a 4' phosphate group linked to the glucosamine backbone. While it is common for the lipid A 1-phosphate group to cleave from the molecule during either the mild acid hydrolysis step of lipid A purification or in the mass spectrometer, the 4' phosphate group is not susceptible to such cleavage (25). 4'-dephosphorylated lipid A species are found in other organisms, such as *Helicobacter pylori* and *Francisella tularensis*, due to the activity of lipid A 4' phosphatases (50, 51). Although a *P. aeruginosa* lipid A 4'-phosphatase has not been identified, *P. aeruginosa* has phosphatidylglycerolphosphate phosphatase orthologs. As these phosphatases can remove a phosphate group from phosphatidylglycerolphosphate and leave behind a free hydroxyl group, it is possible that one of these proteins is responsible for removal of the 4' phosphate group (88). We also cannot rule out the possibility that $LpxT_{Pa}$ itself is acting as a lipid A 4' phosphatase. The existence of this unusual lipid A species further demonstrates the need for a deeper understanding of lipid A modification systems in *P. aeruginosa*.

We have also found that $LpxT_{Pa}$ activity is inhibited by growth in limiting Mg^{2+} independently of *lpxT*$_{Pa}$ transcription. While TLC and MALDI-TOF analysis of lipid A from bacteria grown in low Mg^{2+} demonstrated no phosphate group addition to lipid A (Fig. 2.8A and B), our quantitative PCR data revealed no significant change in *lpxT*$_{Pa}$ transcription in response to limiting Mg^{2+} (Fig. 2.8C). Instead, low Mg^{2+} induces transcription of *arnT* and *pagP* resulting in addition of L-Ara4N and palmitate, respectively (Fig. 2.8C and 2.9) (19, 67). However, if *arnT* is deleted from the genome, $LpxT_{Pa}$ remains partially active even when Mg^{2+} is limiting (Fig. 2.10). While *tris*-phosphorylated lipid A species are present in the PA14Δ*arnT* mutant, this species is clearly diminished when cells are grown in low Mg^{2+} compared to growth in high Mg^{2+} (Fig. 2.10A and B). This strongly suggests that the absence of *tris*-phosphorylated species in limiting Mg^{2+} is not merely due to competition from ArnT. Instead, Mg^{2+}-dependent reduction of $LpxT_{Pa}$ activity occurs independently from ArnT activity. By altering the expression or activity of these two modification enzymes, *P. aeruginosa* has evolved a coordinated mechanism that mediates appropriate alterations in the outer membrane to promote survival in a changing environment.

This report demonstrates another level of control involved in environmental adaptation that can contribute to *P. aeruginosa*'s ability to persist as a pathogen. While $LpxT_{Pa}$-dependent lipid A phosphorylation has not yet been definitively linked to an increase in cell survival under a specific environmental condition, a new study has revealed that in a *P. aeruginosa* burned mouse acute infection model, an *lpxT*$_{Pa}$ mutant was significantly less fit within the burn wound relative to analysis in succinate growth

medium (90). Further investigation is necessary to determine the role of *lpxT*$_{Pa}$ in acute *P. aeruginosa* infections.

Although we have begun to characterize LpxT$_{Pa}$ regulation, future work is required to determine which regulatory system or systems might be involved in Mg^{2+}-dependent modulation of LpxT$_{Pa}$ activity. Furthermore, we have not tested whether any other lipid A modification activation signals (i.e. cationic antimicrobial peptides) inhibit LpxT$_{Pa}$ activity as well. In *P. aeruginosa* both PhoPQ and PmrAB are independently activated by limiting Mg^{2+}, while the latter system can also be activated by antimicrobial peptides such as polymyxin (67, 68). In *E. coli* and *S. enterica*, inhibition of LpxT activity is PmrA-dependent. Upon activation by inducing environmental conditions, PmrA acts as a transcription factor to regulate many target genes. One such gene encodes the small regulatory protein PmrR, which interacts with LpxT to prevent its function (74). While PmrR orthologs exist in other enteric bacteria, including *Citrobacter koseri* and *Klebsiella pneumonia*, no such ortholog is present in microorganisms such as *P. aeruginosa* and *N. meningitidis* (74). It is therefore likely that a distinct regulatory mechanism from *E. coli* and *S. enterica* is involved in LpxT regulation in other Gram-negatives like *P. aeruginosa*. Further investigation of regulatory mechanisms involved in modulation of LpxT$_{Pa}$ activity and of *P. aeruginosa* lipid A remodelling in general is needed to better understand how this formidable pathogen adapts to its surroundings.

Chapter 3: Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system[2]

3.1 INTRODUCTION

Modification of the canonical, hexa-acylated, *bis*-phosphorylated lipid A molecule produced in Gram-negatives (Fig. 1.2A; black) alter its chemical properties to bolster membrane integrity. A repertoire of modification enzymes is responsible for the dynamic structure of *P. aeruginosa* lipid A. Previous work from our laboratory recently revealed that *P. aeruginosa* has an LpxT lipid A kinase that adds an additional phosphate group to the 1- or the 4' position under standard laboratory growth conditions (Fig. 1.2A; brown)(31). Hydroxylation of the secondary acyl chains can also occur by one of two LpxO enzymes, although the purpose for this modification remains to be elucidated (Fig. 1.2B; orange) (91). In addition to these modifications, the toxicity of *P. aeruginosa* lipid A can be affected by altering the acylation pattern due to activity of the PagL deacylase or the PagP palmitoyl-transferase (Fig. 1.2B; pink and green) (6, 19, 25, 34). Aside from influencing endotoxicity, lipid A modifications can contribute to antimicrobial peptide resistance (2). Addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to either phosphate group of *P. aeruginosa* lipid A by the enzyme ArnT is one such strategy (Fig. 1.2B; blue)(18, 33). Palmitoylation has also been shown to increase antimicrobial resistance (19).

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[2] Large portions of this chapter have been previously published (copyright by John Wiley and Sons, re-used with permission). **Nowicki EM, O'Brien JP, Brodbelt JS, Trent MS**. 2015. Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system. Mol Microbiol. doi: 10.1111/mmi.13018. (E.M.N. designed and performed experiments and wrote the manuscript under the supervision of M.S.T.; J.P.O. designed and performed the ESI and UVPD-MS experiments under the supervision of J.S.B.).

In Gram-negatives such as *E. coli*, *Helicobacter pylori*, *Campylobacter jejuni*, and *Neisseria gonorrhoeae* addition of phosphoethanolamine (pEtN) groups to lipid A by the enzyme EptA also promotes polymyxin resistance and virulence (42, 44–47, 51). Although *P. aeruginosa* has pEtN transferase orthologs, pEtN addition has not been observed in *P. aeruginosa* lipid A prepared from cells grown under conditions that induce this modification in other organisms or from *P. aeruginosa* clinical isolates. We investigated whether or not *P. aeruginosa* lipid A could be modified with pEtN, and if so, under what conditions.

Here we report that *P. aeruginosa* gene PA14_39020 is a functional pEtN transferase which we have named $EptA_{Pa}$ that strictly modifes the 4' phosphate group of lipid A. We also demonstrate that zinc acts as a signal to induce $eptA_{Pa}$ transcription via the ColRS two-component system (Fig. 3.12). While transcription of $eptA_{Pa}$ is upregulated in response to Zn^{2+}, *arnT* transcription is downregulated, suggesting that mechanisms are in place to mediate strict control over specific lipid A modifications. The existence of $eptA_{Pa}$ reveals the potential for greater diversity in *Pseudomonas* lipid A structure and the versatility of the outer membrane.

3.2 RESULTS

3.2.1 *P. aeruginosa* has a functional EptA enzyme

In silico analysis identified three *P. aeruginosa eptA* orthologs with significant identity to the *S. enterica eptA* (*pmrC*) ortholog (40). These include PA14_58610 (24% identity, E-value 3e-28), PA14_21210 (43%, E-value 1e-149) and PA14_39020 (43%, E-

value 6e-149). Since pEtN-modified *P. aeruginosa* lipid A has not been previously reported, we tested whether these orthologs could function as a lipid A pEtN transferase by expressing each gene *in trans* in the *E. coli eptA* mutant (W3110Δ$eptA_{Ec}$). ^{32}P-labeled lipid A was prepared and separated by TLC from these and relevant control strains including wild-type strain W3110 and W3110Δ$eptA_{Ec}$+empty vector.

While no pEtN is detected in lipid A prepared from W3110 or W3110Δ$eptA_{Ec}$ + empty vector (Fig. 3.1A, lanes 1 and 2), expression of both $eptA_{Ec}$ and PA14_39020 resulted in pEtN-modified lipid A (Fig. 3.1A, lanes 3 and 6). MALDI-TOF mass spectrometry (MS) analysis also confirmed that while W3110Δ$eptA_{Ec}$ expressing empty vector had no pEtN (Fig. 3.1B), lipid A prepared from W3110Δ$eptA_{Ec}$+PA14_39020 (p$eptA_{Pa}$) was modified with pEtN, as evidenced by the ion of m/z 1920.4 (Fig. 3.1C; predicted [M-H]- at m/z 1920.2).

Since $EptA_{Pa}$ can add pEtN to *E. coli* lipid A, we next determined whether $EptA_{Pa}$ modified *P. aeruginosa* lipid A. ^{32}P-labeled lipid A from wild-type strain PA14, PA14+empty vector or PA14+$eptA_{Pa}$ was analyzed. TLC separation of lipid A clearly demonstrated an altered profile of PA14 expressing p$eptA_{Pa}$ (Fig. 3.2A, lane 3) relative to PA14 or PA14+empty vector (Fig. 3.2A, lanes 1 and 2). Penta-acylated, palmitoylated and L-Ara4N-modified lipid A species were observed by MALDI-TOF MS analysis of lipid A isolated from PA14+empty vector (Fig. 3.2B). Whereas expression of $eptA_{Pa}$ in PA14 resulted in abundant ions of m/z 1489.0 and 1727.3 which correspond to the pEtN-modified lipid A species (Fig. 3.2C; predicted [M-H]- at m/z 1489.9 and 1728.1,

respectively). These results reveal that *P. aeruginosa* has a functional lipid A pEtN-transferase enzyme capable of modifying both *E. coli* and *P. aeruginosa* LPS.

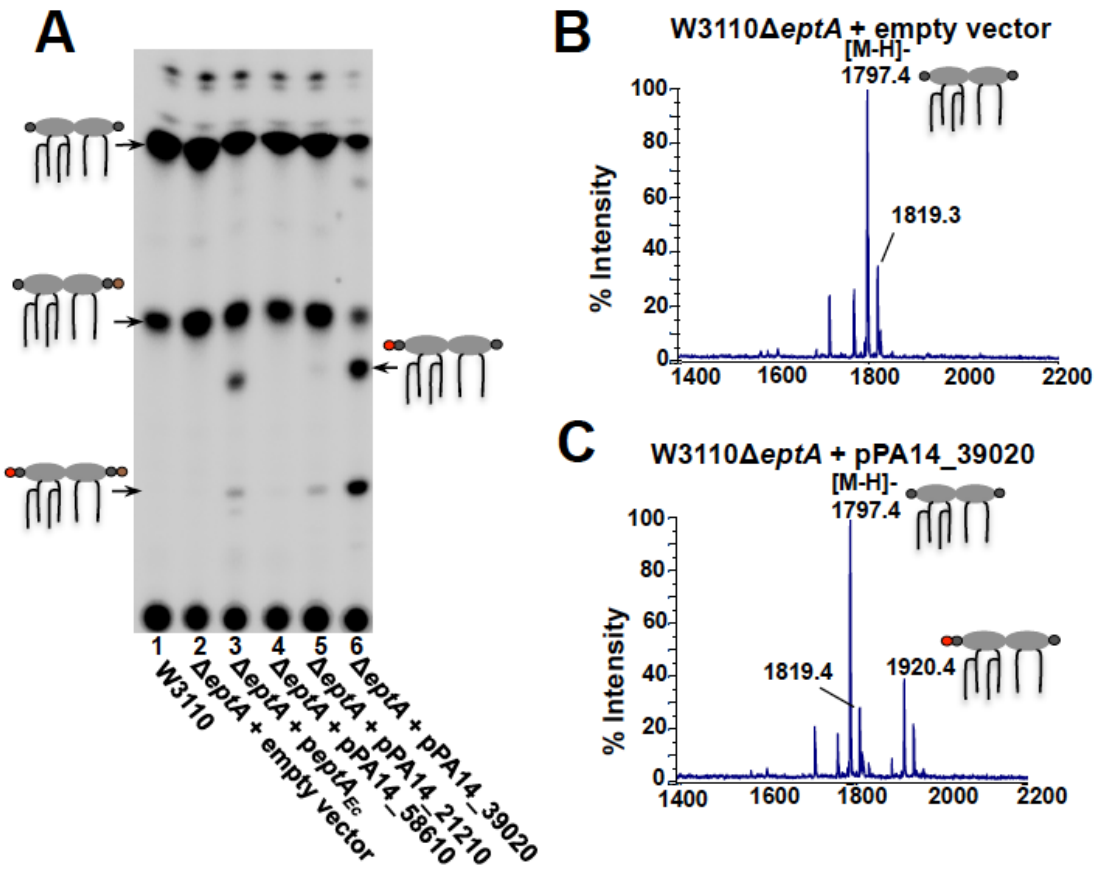

Figure 3.1: Identification of a functional *P. aeruginosa* pEtN transferase ($eptA_{Pa}$) in *E. coli*.

A) *P. aeruginosa eptA* orthologs were heterologously expressed in K-12 *E. coli* strain W3110Δ*eptA*. PA14_39020 and $eptA_{Ec}$ were able to modify lipid A. B) MALDI-TOF MS analysis of lipid A isolated from Δ*eptA* + empty vector shows no pEtN addition to lipid A, while (C) expression of PA14_39020 reveals pEtN modification.

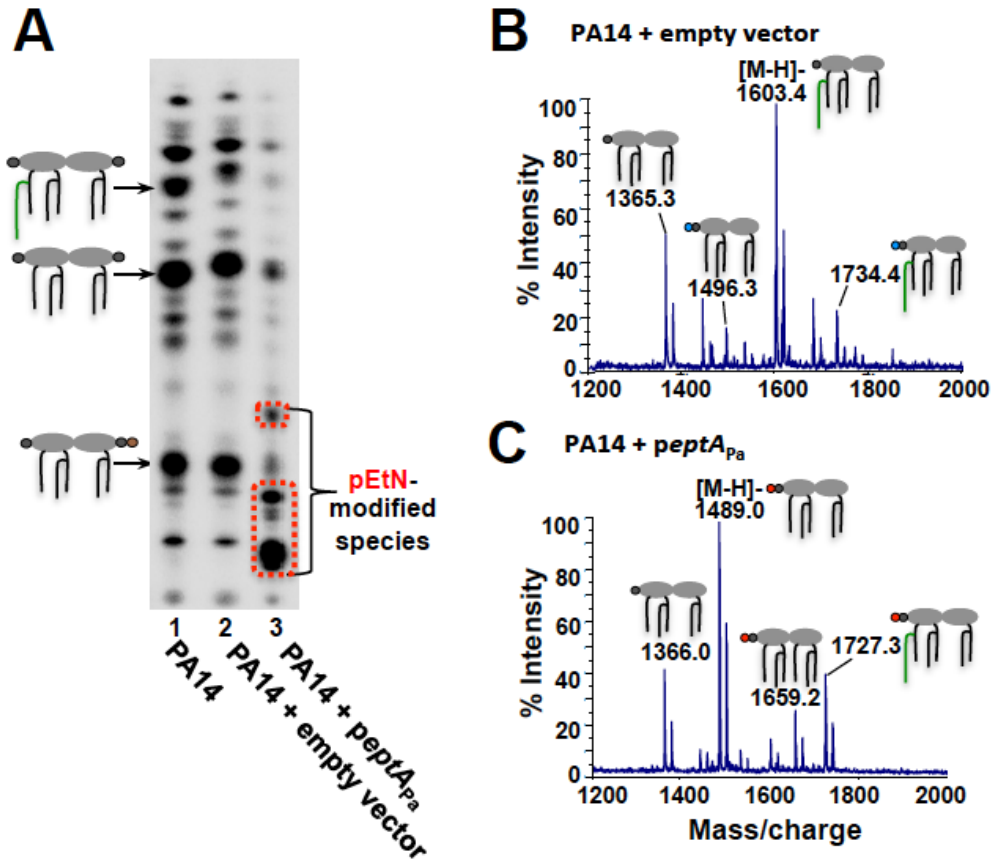

Figure 3.2: Heterologous expression of a *P. aeruginosa eptA* ortholog results in pEtN addition to the lipid A.

A) Cells were grown in MOPS minimal medium. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Expression of PA14_39020 (*eptA*$_{Pa}$) in *P. aeruginosa* results in modified lipid A species. B) MALDI-TOF MS analysis of PA14 + empty vector grown in MOPS minimal medium reveals no pEtN addition to the molecule, while C) analysis of PA14 + p*eptA*$_{Pa}$ shows pEtN modification of the lipid A. The fractions most representative of pEtN modification are shown.

3.2.2 $EptA_{Pa}$ adds pEtN strictly to the lipid A 4' phosphate group

Previous work from our laboratory has demonstrated that in *P. aeruginosa, S. enterica* and *E. coli* the position of lipid A modification can be important due to potential competition with other modification groups. For example in *S. enterica* and *E. coli* EptA preferentially adds pEtN to the lipid A 1-phosphate group, which is the sole site of phosphorylation by the kinase LpxT (42). Environmental conditions that activate *eptA* transcription simultaneously inhibit LpxT activity to prevent competition (42, 74). However, since both LpxT and ArnT enzymes in *P. aeruginosa* can act on either lipid A phosphate group (31, 33) we questioned whether $EptA_{Pa}$ also has dual positional activity. To determine this we removed the 1- or the 4'-phosphate group of lipid A by heterologous expression of *Fransicella novicida* LpxE or LpxF phosphatases (49, 50), respectively, and tested the ability of $EptA_{Pa}$ to modify lipid A. This experiment was done in *E. coli* strain BN2 (4) since its lipid A is penta-acylated, and LpxF can only act on penta-acylated lipid A (50). BN2 also lacks some lipid A modification machinery including LpxT to facilitate easier analysis of the lipid A profiles.

Expression of $eptA_{Pa}$ resulted in a pEtN-modified lipid A species that migrated below the unmodified *bis*-phosphorylated species (Fig. Fig. 3.3A, lane 2). Expression of either *lpxE* or *lpxF* caused a marked increase of mono-phosphorylated lipid A, which migrates near the top of the TLC plate (Fig. 3.3A, lanes 3 and 5). Simultaneous expression of $eptA_{Pa}$ and *lpxE* resulted in the appearance of a species that migrated a distance between that of unmodified *bis*-phosphorylated lipid A and pEtN-modified lipid

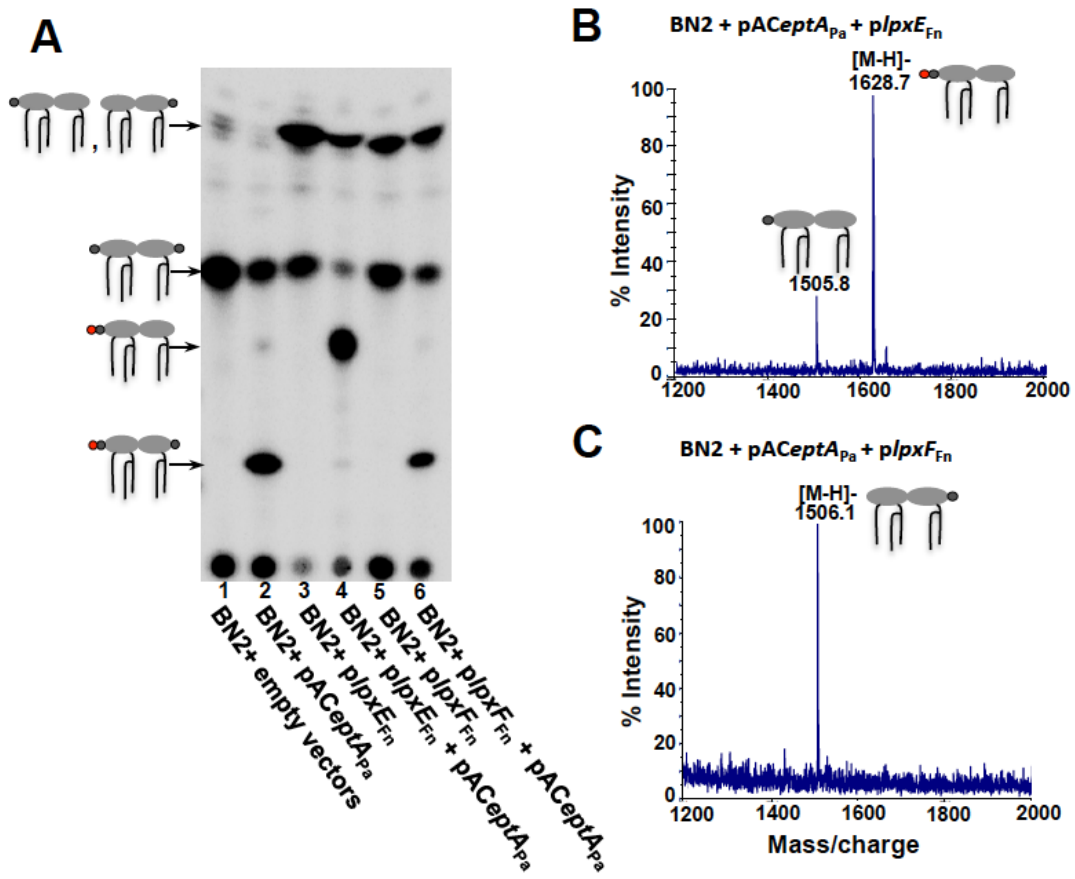

Figure 3.3: EptA$_{Pa}$ adds pEtN exclusively to the lipid A 4' phosphate group.

A) Cells were grown in LB broth. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Heterologous expression of *eptA*$_{Pa}$ in BN2 results in a pEtN-modified species, while expression of either the *lpxE*$_{Fn}$ or *lpxF*$_{Fn}$ phosphatase results in an increased of monophosphorylated species. Co-expression of *lpxE*$_{Fn}$ and *eptA*$_{Pa}$ results in pEtN addition to the 1-dephosphorylated lipid A molecule, while no pEtN addition of 4' – dephosphorylated species is detected. B) MALDI-TOF analysis of lipid A isolated from BN2 coexpressing *lpxE*$_{Fn}$ and *eptA*$_{Pa}$ corroborates the presence of a monophosphorylated, pEtN-modified species. C) MALDI-TOF analysis of lipid A isolated from BN2 coexpressing *lpxF*$_{Fn}$ and *eptA*$_{Pa}$ reveals that when the 4' phosphate group is removed, pEtN addition does not occur.

A (Fig. 3.3A, lane 4). MALDI-TOF MS confirmed this species to be pEtN-modified mono-phosphorylated lipid A (Fig. 3.3B). This same pEtN-modified species was not detected when LpxF was expressed with *eptA*$_{Pa}$ (Fig 3.3A, lane 6). The lack of pEtN addition to 4'-dephosphorylated lipid A was further confirmed by MALDI-TOF MS as simultaneous expression of EptA$_{Pa}$ and LpxF resulted in only a single ion of m/z 1506.1, corresponding to mono-phosphorylated lipid A (Fig. 3.3C; predicted [M-H]- at m/z 1507.1).

EptA$_{Pa}$-dependent addition of pEtN to the 4' phosphate group of BN2 and PA14 lipid A was corroborated by ultraviolet photodissociation (UVPD) tandem MS (Figs. 3.4, 3.5 and 3.6). For all UVPD mass spectra, cleavage sites **7** and **8** provided evidence of the presence of a pEtN group. In addition, the glycosidic and cross-ring cleavages at cleavage sites **10** and **11** in the fragmentation map shown in Figure 3.4, sites **9-12** in Figure 3.5, and sites **9-12** and **17-21** in Figure 3.6 further support the location of the pEtN modification at the 4′ phosphate group of each lipid A species. Taken together, these results demonstrate that EptA$_{Pa}$ functions strictly at the 4′ phosphate group, unlike any previously characterized EptA enzyme.

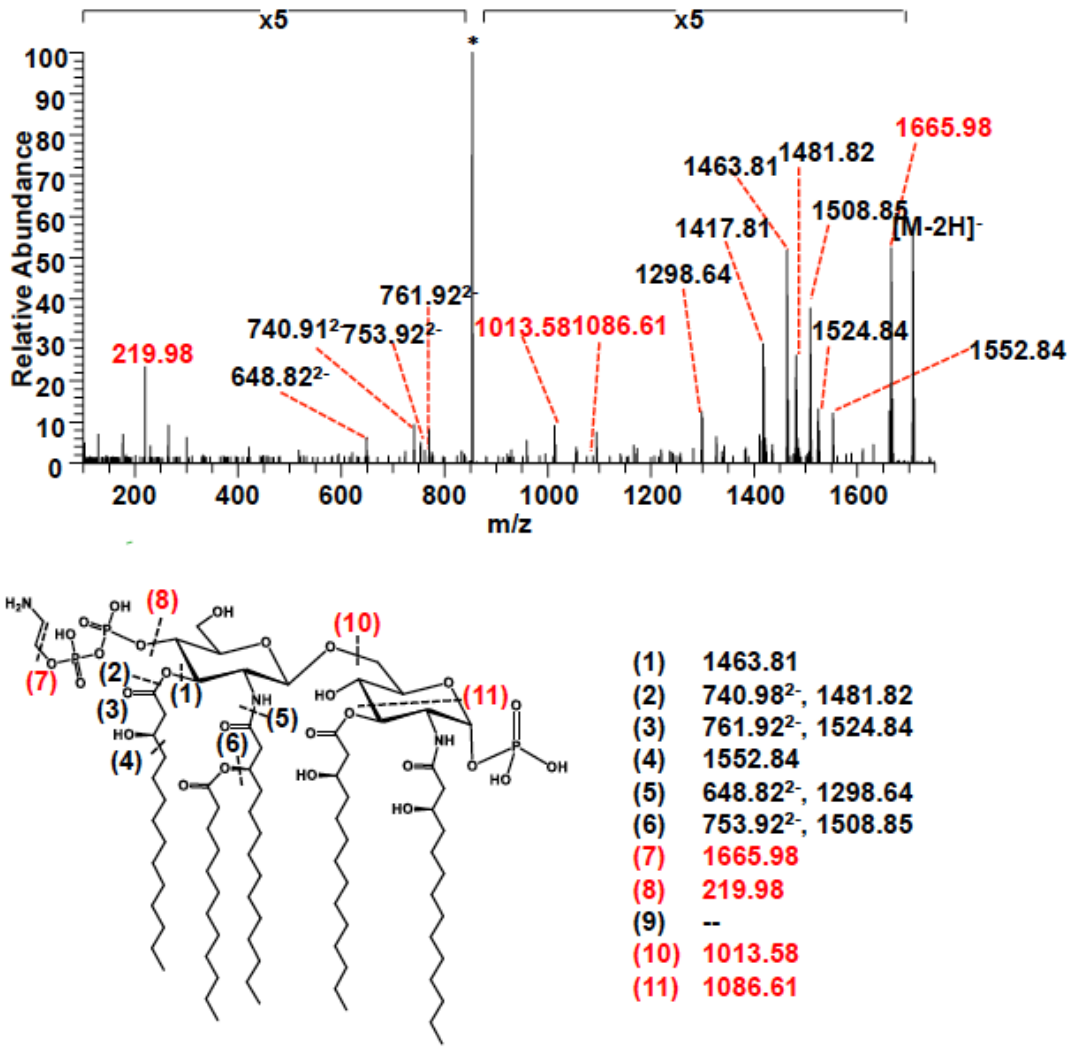

Figure 3.4: UVPD mass spectrum and fragmentation map of doubly deprotonated lipid A prepared from BN2 + p*eptA*$_{Pa}$ [M_r = 1710.03].

Key fragments and cleavage sites that support the identification and location of the 4′ modification are shown in red font. The precursor ion is marked with an asterisk.

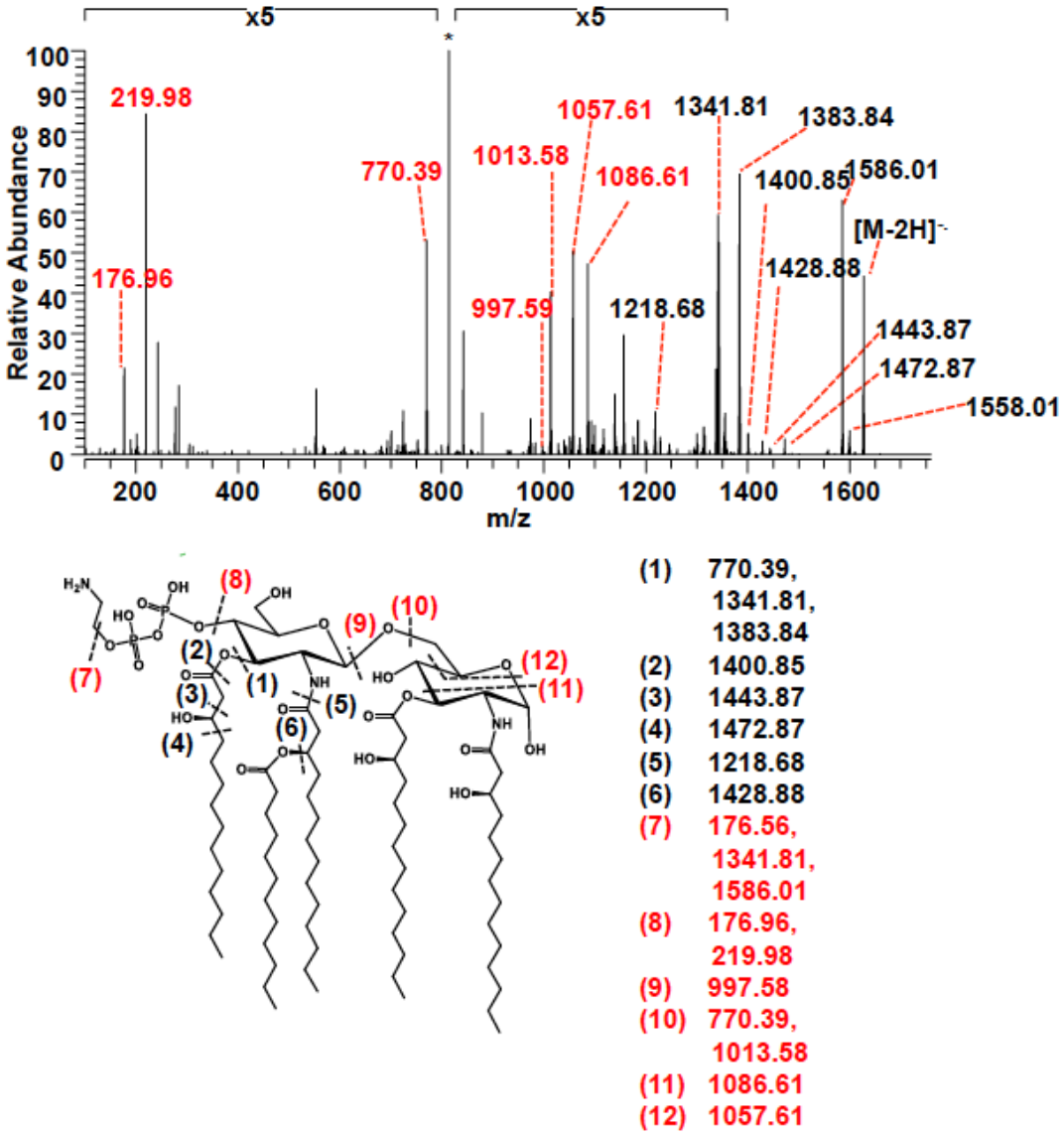

Figure 3.5: UVPD mass spectrum and fragmentation map of doubly deprotonated lipid A prepared from BN2 + p*eptA*$_{Pa}$ + p*lpxE* [M_r = 1630.06].

Key fragments and cleavage sites that support the identification and location of the 4′ modification are shown in red font. The precursor ion is marked with an asterisk.

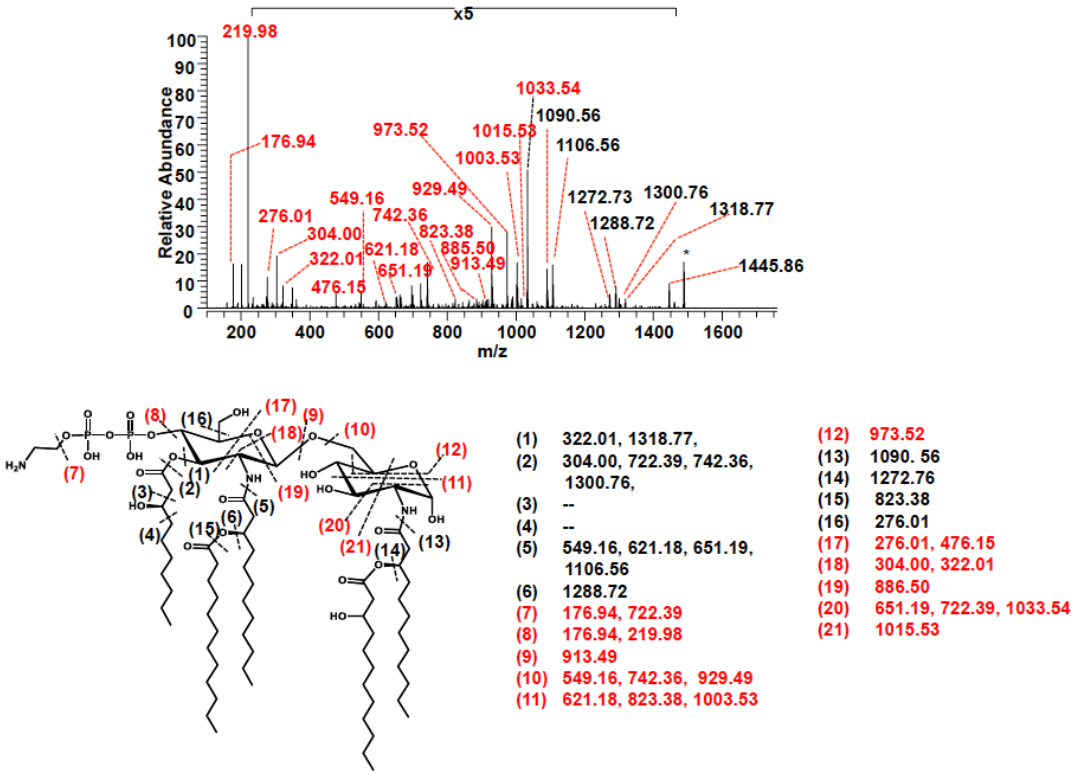

Figure 3.6: UVPD mass spectrum and fragmentation map of singly deprotonated lipid A prepared from PA14 + peptAPa [M_r = 1489.91].

Key fragments and cleavage sites that support the identification and location of the 4′ modification are shown in red font. The precursor ion is marked with an asterisk.

3.2.3 Extracellular zinc induces pEtN addition to lipid A

In *S. enterica,* modification of lipid A with pEtN is induced via the PmrAB two-component system in response to mildly acidic pH (57), and indirectly via the PhoPQ system when Mg^{2+} is limiting or cationic antimicrobial peptides are present (62, 92). Since none of these signals induced pEtN addition to *P. aeruginosa* lipid A (data not shown), we next tested transition metals including Fe^{3+} and Zn^{2+}, which activate PmrAB in *E. coli* (59, 61). We also tested Ga^{3+} due to its chemical similarity to Fe^{3+}, and Cd^{2+}, which is closely related to Zn^{2+} (93, 94). Transition metals Co^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+} were also tested for their ability to induce pEtN modification of lipid A since they are associated with biological catalysts and are commonly found in the environment (95, 96).

Lipid A was isolated from ^{32}P-labeled *P. aeruginosa* grown in LB alone or supplemented with metal. Addition of extracellular Zn^{2+} but no other metal tested resulted in modified lipid A (Fig. 3.7A, lane 3). Zn^{2+}-dependent modification was abolished when $eptA_{Pa}$ was deleted from the genome and restored upon complementation with the native $eptA_{Pa}$ promoter (Figs. 3.7A, lanes 4 and 5), suggesting that the changes observed were due to $EptA_{Pa}$ activity. To determine whether Zn^{2+} induced transcription of $eptA_{Pa}$, cDNA was prepared from cells grown in the presence or absence of Zn^{2+}. As shown by both quantitative and semi-quantitative reverse-transcriptase (RT) PCR, $eptA_{Pa}$ gene expression is induced when Zn^{2+} is added to the growth media (Figs. 3.7B and 3.8). These results indicate that transcription of $eptA_{Pa}$ is dependent on extracellular Zn^{2+}.

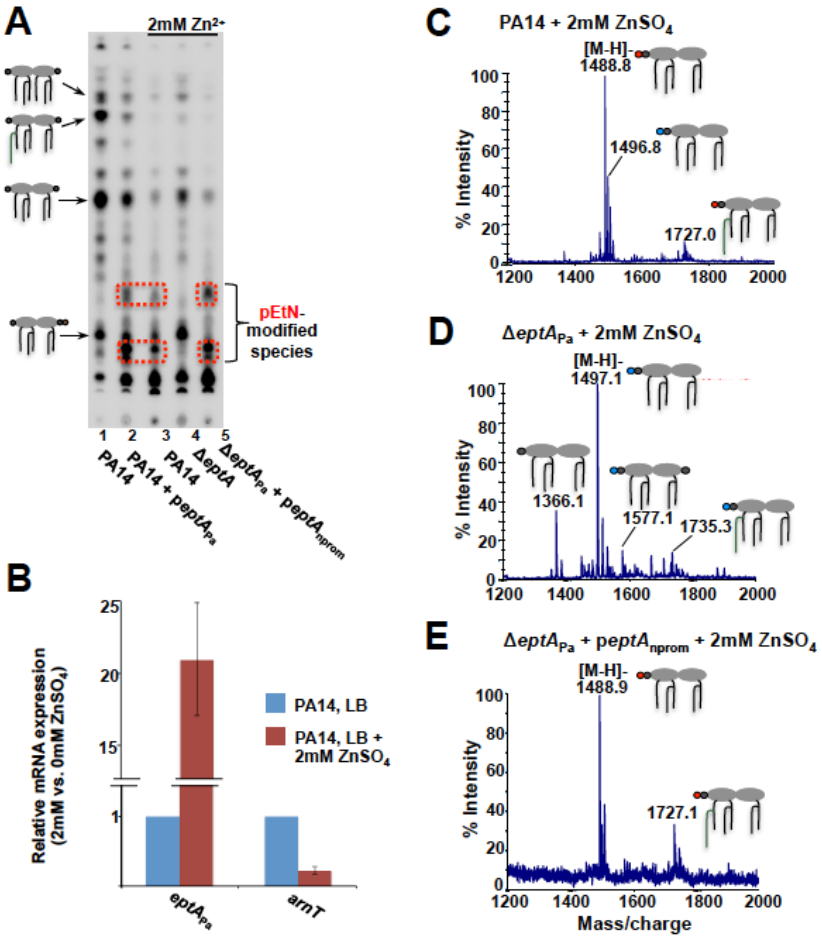

Figure 3.7: Zn^{2+} induces transcription of *eptA*$_{Pa}$.

A) Cells were grown in LB broth. Major ^{32}P-labeled lipid A species are indicated with a cartoon corresponding to the lipid A structure; colors of modification groups are the same as those used in Fig. 1. Both heterologous expression of *eptA*$_{Pa}$ as well as addition of 2mM $ZnSO_4$ to the media results in pEtN addition to lipid A. This modification is not detectable in the *eptA*$_{Pa}$ mutant, but restored upon complementation with p*eptA*$_{nprom}$. B) Relative gene expression of *eptA*$_{Pa}$ and *arnT* in response to Zn^{2+}. Transcription of *eptA*$_{Pa}$ is induced by 2mM $ZnSO_4$ approximately 21-fold. Zn^{2+} downregulates *arnT* transcription by >4-fold. Ratios were standardized relative to expression of the housekeeping control gene, *clpX*. C), D) and E). MALDI-TOF MS analysis of lipid A prepared from cells grown in LB broth. C) Analysis of PA14 + 2mM $ZnSO_4$ reveals pEtN addition to lipid A, while D) Δ*eptA*$_{Pa}$ + 2mM $ZnSO_4$ shows no pEtN modification, but instead L-Ara4N addition. E) Complementation of Δ*eptA*$_{Pa}$ with p*eptA*$_{nprom}$ restores pEtN addition to the lipid A in response to Zn^{2+}. The fractions most representative of pEtN modification are shown.

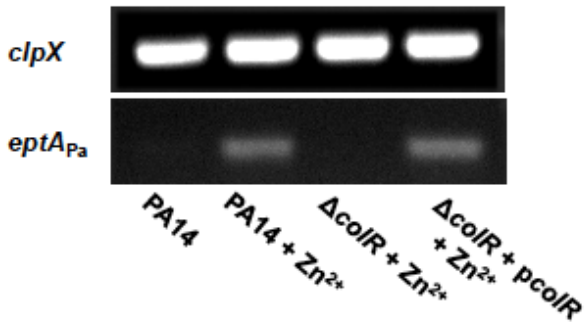

Figure 3.8: Excess zinc induces transcription of *eptA*$_{Pa}$.

Semi-quantitative RT-PCR shows no detectable *eptA*$_{Pa}$ transcription in PA14 cells grown in LB alone, while addition of 1mM $ZnSO_4$ results in detectable transcription. No transcription of *eptA*$_{Pa}$ is visualized in PA14Δ*colR* grown in LB with 1mM $ZnSO_4$. When this mutant is complemented, *eptA*$_{Pa}$ transcription in response to 1mM $ZnSO_4$ can once again be seen. The stably expressed housekeeping gene *clpX* was used as a control.

To confirm whether Zn^{2+}-dependent induction of $EptA_{Pa}$ synthesis resulted in pEtN addition to *P. aeruginosa* lipid A, MALDI-TOF MS analysis of lipid A isolated from cells grown with or without Zn^{2+} was performed. Wild-type *P. aeruginosa* lipid A was modified with pEtN in the presence of Zn^{2+} (Fig. 3.7C), while lipid A prepared from the PA14Δ$eptA_{Pa}$ mutant showed no pEtN modification when Zn^{2+} was added to the media (Fig. 3.7D). Complementation of PA14Δ$eptA_{Pa}$ using the native $eptA_{Pa}$ promoter restored Zn^{2+}-dependent pEtN addition to the lipid A (Figs. 3.7E). MS analysis revealed that in addition to pEtN addition, L-Ara4N-modified lipid A was present in PA14 grown with Zn^{2+} (Fig. 3.7C). We were therefore curious as to how Zn^{2+} might influence L-Ara4N addition to lipid A. Since Zn^{2+} affected the transcription of $eptA_{Pa}$, we assessed whether Zn^{2+} altered *arnT* gene expression by performing quantitative RT-PCR. While $eptA_{Pa}$ transcription increased by 21-fold in the presence of 2mM Zn^{2+}, *arnT* transcription was downregulated >4-fold (Fig. 3.7B). This result indicates that pEtN modification is selected for in the presence of Zn^{2+} while *arnT* expression, and thus L-Ara4N modification of lipid A, is downregulated.

3.2.4 The ColRS two-component system induces pEtN addition to lipid A

Since inducible lipid A modification genes like *eptA* are commonly regulated by two-component systems, our next goal was to determine the system responsible for $eptA_{Pa}$ transcriptional activation in response to Zn^{2+}. The ColRS system has recently been shown to respond to transition metals including Zn^{2+} (72). Our first approach was therefore to investigate the presence of potential ColR binding sites in the $eptA_{Pa}$

promoter. A consensus ColR binding site has been determined for promoters of genes within the ColR regulon in *P. putida* (97). Using the Virtual Footprint online analysis tool (98), we found three potential ColR binding sites within the $eptA_{Pa}$ promoter region with close agreement to this consensus sequence (Fig. 3.9A), suggesting that ColR binds to the $eptA_{Pa}$ promoter. It was then tested whether overexpression of *colR* could induce $eptA_{Pa}$ transcription by semi-quantitative RT-PCR of cDNA. Response regulators *pmrA* and *phoP*, which directly or indirectly regulate *eptA* transcription in *S. enterica* were also tested. Only overexpression of *colR* resulted in detectable transcription of $eptA_{Pa}$ (Fig. 3.9B). Lipid A was modified with pEtN upon overexpression of *colR,* as demonstrated by both TLC separation of ^{32}P-labeled lipid A (Fig. 3.9C, lane 4) and MALDI-TOF MS analysis of PA14 + p*colR* (Fig. 3.9D).

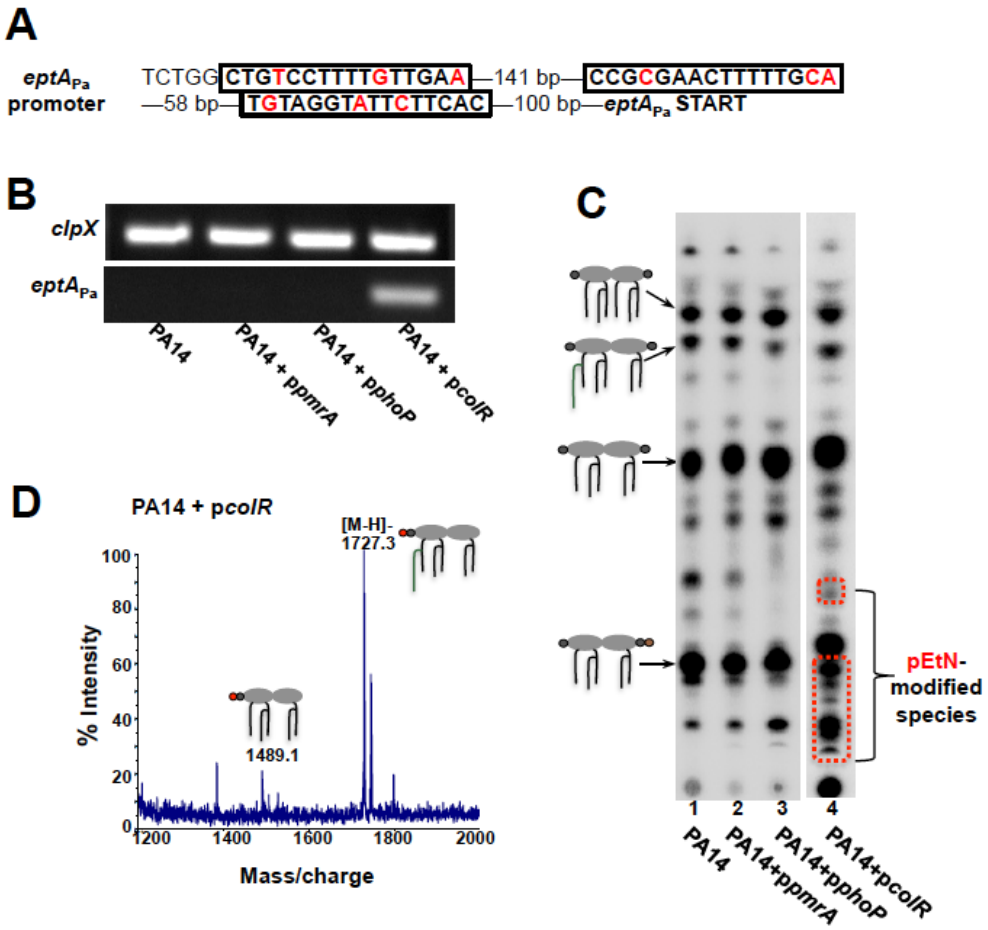

Figure 3.9: The two-component system response regulator ColR activates *eptA*$_{Pa}$ transcription.

A) Putative *eptA*$_{Pa}$ promoter ColR binding sites are in bold and boxed; nucleotides that deviate from the conserved recognition sequence in *P. putida* ((T/C)(T/C)NA(C/G)NN(T/C)TTTTT(C/G)AC) are indicated in red. The number of base pairs between ColR sites or upstream of the start codon is indicated. B) Semi-quantitative RT-PCR of cDNA prepared from cells grown in MOPS minimal medium. While *eptA*$_{Pa}$ is not transcribed in PA14, expression of *colR in trans* results in *eptA*$_{Pa}$ transcription. C) Lipid A was isolated from ^{32}P-labeled cells grown in MOPS minimal medium and separated by TLC. Only expression of the *colR* response regulator, and not *pmrA* or *phoP*, results in pEtN modification of lipid A. D) MALDI-TOF MS analysis of PA14 + p*colR* grown in MOPS minimal medium reveals pEtN-modified lipid A. The fraction most representative of pEtN modification is shown.

To determine whether ColR induction of *eptA*$_{Pa}$ transcription is dependent on Zn^{2+}, a PA14 *colR* deletion mutant was generated and assessed for transcription of *eptA*$_{Pa}$ in the presence or absence of Zn^{2+} by both quantitative and semi-quantitative RT-PCR analysis. Although a Zn^{2+} concentration of 2mM had been used in the initial Zn^{2+} assay experiments, the PA14Δ*colR* mutant was sensitive to 2mM Zn^{2+}. Instead, 1mM Zn^{2+} was used, which was sufficient to visualize pEtN modification in PA14 (Fig. 3.10A, lane 2). Minimal *eptA*$_{Pa}$ transcription was detected in response to Zn^{2+} upon deletion of *colR*; complementation of this mutant restored Zn^{2+}-dependent *eptA*$_{Pa}$ transcription by >4-fold (Fig. 3.10B, Fig. 3.8). While 1mM Zn^{2+} induced pEtN modification of PA14 lipid A (Fig. 3.10A, lane 2), lipid A from the PA14Δ*colR* was not modified with pEtN in response to Zn^{2+}, as determined by both TLC separation of ^{32}P-labeled lipid A and MALDI-TOF MS analysis (Fig. 3.10A, lane 3 and Fig. 3.10C). pEtN addition was restored upon complementation of the *colR* mutant with p*colR*$_{nprom}$ (Fig. 3.10A, lane 4 and Fig. 3.10D). A PA14 *colS* mutant and complemented mutant were also tested for pEtN addition to lipid A in response to Zn^{2+} by TLC separation of ^{32}P-labeled lipid A. As for the *colR* mutant, lipid A modification with pEtN was not detected in the *colS* mutant grown in the presence of 1mM Zn^{2+}, but was restored upon complementation of *colS* with p*colS*$_{nprom}$ (Fig. 3.11, lanes 3 and 4). These results demonstrate that the ColRS system induces pEtN addition to lipid A upon sensing Zn^{2+}.

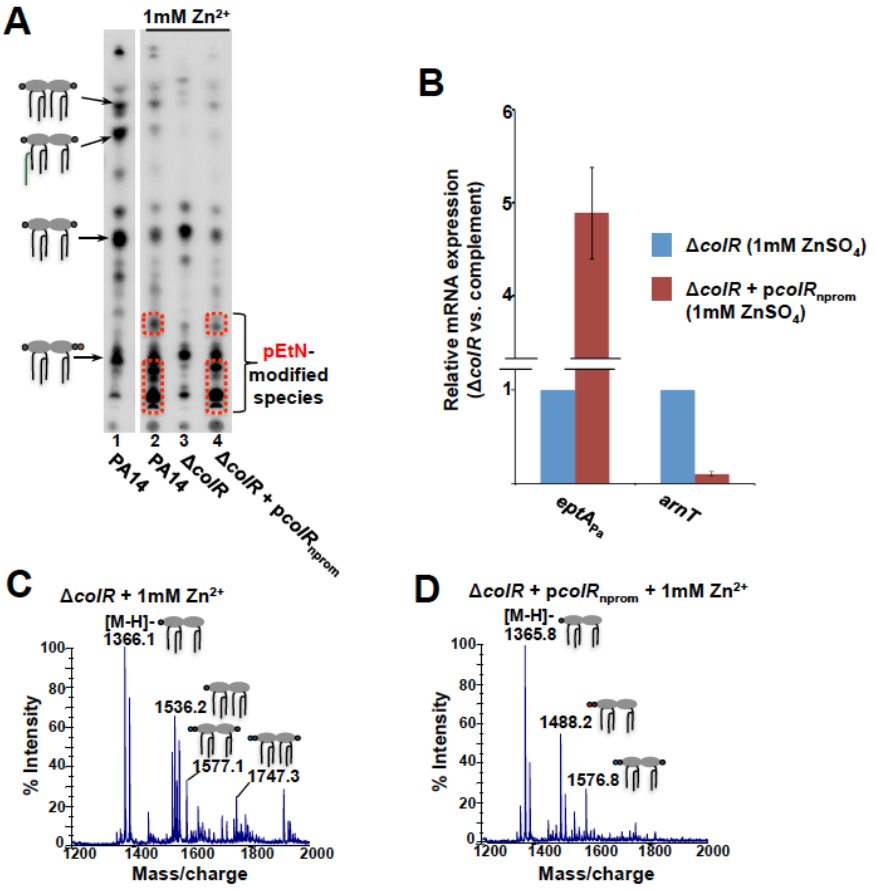

Figure 3.10: Deletion of *colR* results in loss of Zn^{2+}-induced pEtN modification of *P. aeruginosa* lipid A.

A) Lipid A was isolated from ^{32}P-labeled cells grown in LB broth and separated by TLC. While pEtN modification of lipid A is detectable for PA14 + 1mM $ZnSO_4$, no such modification occurs in PA14Δ*colR* in response to Zn^{2+}. Modification is restored in the complemented mutant. B) Relative gene expression of $eptA_{Pa}$ and *arnT* in response to Zn^{2+} in the Δ*colR* mutant or complemented mutant. Transcription of $eptA_{Pa}$ in the presence of 1mM $ZnSO_4$ is induced >4-fold in a ColR-dependent manner. An approximately 10-fold decrease in *arnT* transcription in the presence of 1mM $ZnSO_4$ is also dependent on ColR. Ratios were standardized relative to expression of the housekeeping control gene, *clpX*. C) and D). MALDI-TOF MS analysis of lipid A prepared from cells grown in LB broth. C) No pEtN modification is detected in the PA14Δ*colR* mutant grown in LB + 1mM $ZnSO_4$. D) Complementation of PA14Δ*colR* with p$colR_{nprom}$ restores the Zn^{2+}-dependent pEtN addition to the lipid A. The fractions most representative of pEtN modification are shown.

We also investigated whether the downregulation of *arnT* transcription in the presence of Zn^{2+} was dependent on the ColR response regulator. Gene expression of *arnT* was analyzed by quantitative RT-PCR in the PA14 *colR* mutant and complemented strains in the presence of 1mM Zn^{2+}. Transcription of *arnT* was reduced by approximately 10-fold upon complementation of *colR* (Fig. 3.10B). This result indicates that ColR activates transcription of $eptA_{Pa}$ in response to Zn^{2+} while downregulating *arnT* transcription.

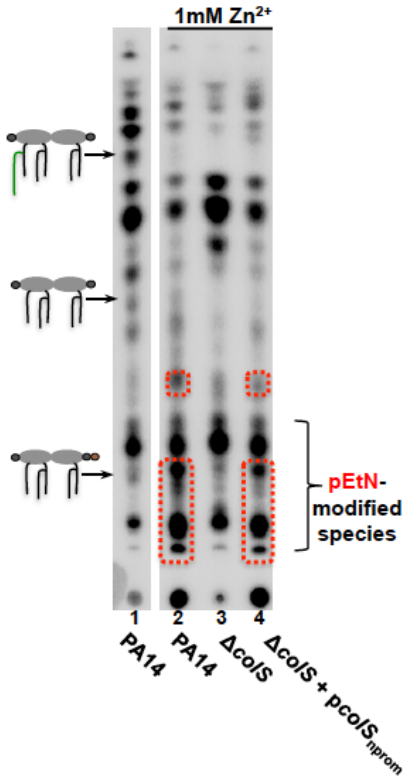

Figure 3.11: Deletion of *colS* results in loss of Zn^{2+}-induced pEtN modification of *P. aeruginosa* lipid A.

Lipid A was isolated from ^{32}P-labeled cells grown in LB broth and separated by TLC. Lipid A species modified with pEtN were observed in PA14 supplemented with 1mM $ZnSO_4$ (lane 2). Deletion of *colS* resulted in no pEtN modification of lipid A in response to Zn^{2+} (lane 3). Modification is restored in the mutant complemented with *colS* (lane 4).

3.3 DISCUSSION

Changes in the environment require bacterial outer membrane remodeling, including LPS structural changes, to promote membrane stability (1). L-Ara4N addition to lipid A phosphate groups contributes to cationic antimicrobial peptide resistance in *P. aeruginosa, E. coli* and *S. enterica* (18, 40, 42). The addition of the amine-containing residue pEtN can also result in increased peptide resistance (42, 44), and in some organisms is a crucial factor for host infection (45, 47). Lipid A modifications in *P. aeruginosa* have been well-studied, yet despite the existence of *eptA* orthologs, pEtN addition has never been observed. Due to the importance of pEtN lipid A modification in other organisms, we investigated the functionality and regulation of *P. aeruginosa eptA* orthologs. In this report, we identify and characterize a functional *P. aeruginosa* lipid A pEtN transferase and determine that Zn^{2+} induces transcription of $eptA_{Pa}$ via the *Pseudomonas*-specific ColRS system (Fig. 3.12).

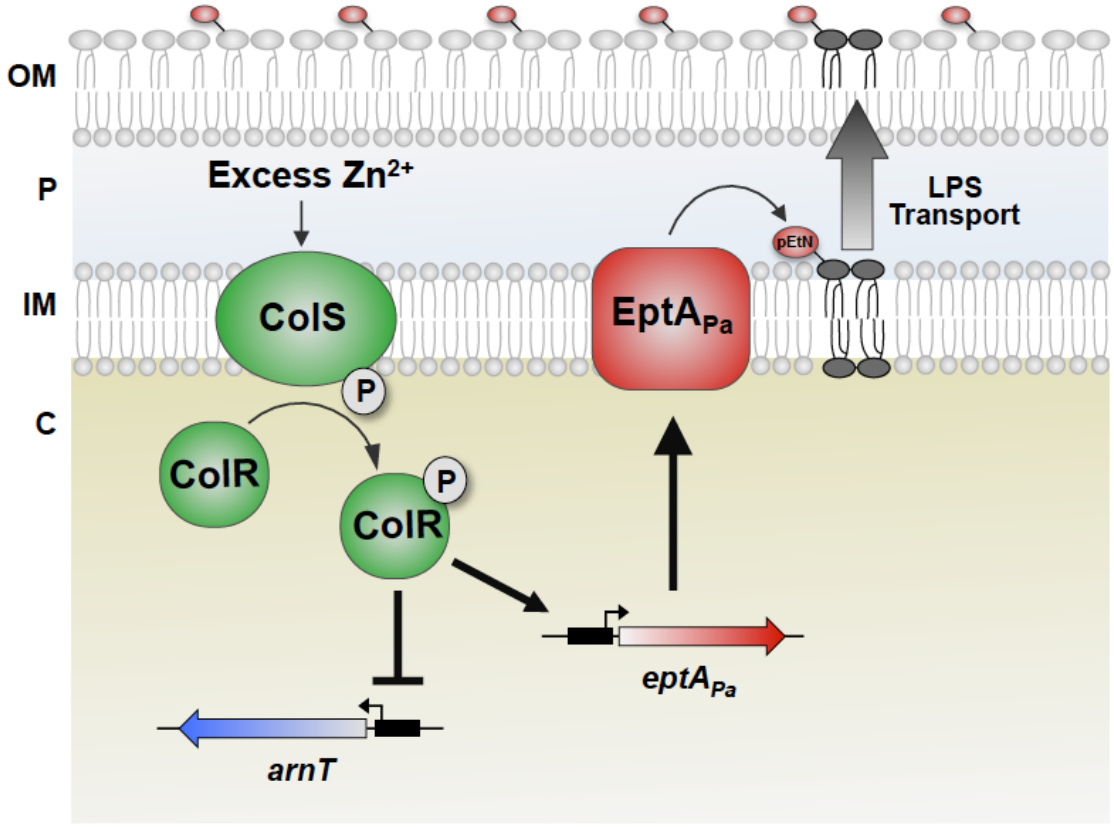

Figure 3.12: Proposed model of pEtN addition to *P. aeruginosa* lipid A.

Upon sensing excess Zn^{2+}, the ColS sensor kinase (green) autophosphorylates and transfers a phosphate group to the response regulator ColR (green). ColR then acts as a transcription factor, inducing transcription of $eptA_{Pa}$ (red) while inhibiting that of *arnT* (blue). $EptA_{Pa}$ protein is synthesized and transfers pEtN to the 4′ phosphate group of lipid A in the inner membrane. Lipid A is then transported to the bacterial cell surface. Following transport to the outer membrane, the 3-hydroxydecanoate acyl chain is removed by PagL (not shown). Cellular components are labelled as follows: OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm).

Overexpression of three *P. aeruginosa eptA* orthologs in *E. coli* revealed that PA14_39020 ($eptA_{Pa}$) was able to modify lipid A with pEtN (Fig. 3.1A and C). It is likely that the other two orthologs add pEtN to other targets in the cell. Based on its homology to *S. enterica* CptA, PA14_58610 may be the enzyme responsible for adding pEtN to the core of *P. aeruginosa* LPS (43, 99). While a very minor amount of lipid A modification is detected by TLC separation of lipid A upon expression of PA14_21210 in PA14, lipid A is probably not the primary target of this enzyme. It is possible that this enzyme modifies an as yet unidentified target, as pEtN transferase enzymes in other organisms have been shown to modify structural proteins of the flagellum and pilus (46, 100), and in doing so has some very minor, non-specific activity toward lipid A. This activity toward lipid A, however, is so minor that is cannot be detected by mass spectrometry analysis (data not shown).

We characterized the site-specificity of pEtN addition to lipid A due to the potential for competition with other modification groups. Whereas pEtN addition occurs specifically or preferentially at the 1-phosphate group of lipid A in *H. pylori* (101) and *S. enterica* (42), respectively, analysis of $EptA_{Pa}$ activity in *E. coli* revealed that this enzyme acts solely at the 4' position (Figs. 3.3, 3.4 and 3.5). $EptA_{Pa}$ activity thus differs from ArnT and LpxT enzymes in *P. aeruginosa* that can modify either lipid A phosphate group (31, 33).

Investigation of conditions that induce pEtN modification revealed that excess Zn^{2+} acts as the activating signal for $eptA_{Pa}$ transcription (Fig. 4.7A, B, and C). *Pseudomonas* species are readily found in the soil and aqueous environments which can

be contaminated with metals due to waste runoff from mines, smelting, and other industrial facilities (95, 102, 103). In such environments, *Pseudomonas* can be exposed to high levels of metal pollutants and has thus evolved the ability to alter gene expression to promote metal tolerance (12, 71, 104, 105). Excess Zn^{2+} may also be relevant in healthcare settings as concentrations up to 1mM can leach out from latex catheters and gloves (104, 106). Deletion of $eptA_{Pa}$, however, does not result in increased sensitivity to Zn^{2+} or to Cd^{2+}, Ga^{3+}, Fe^{3+}, Co^{2+}, Cu^{2+}, Mn^{2+} or Ni^{2+}. Under laboratory settings Zn^{2+}-induced pEtN addition to lipid A has no effect on polymyxin resistance, biocide tolerance, or biofilm formation (data not shown). As the ColRS system was previously implicated to play a role in polymyxin resistance, unidentified genes other than $eptA_{Pa}$ within the ColRS regulon are likely involved in this resistance. The fact that *P. aeruginosa* has evolved regulatory mechanisms to control pEtN addition to lipid A, however, suggests the importance of this modification for conditions we have not yet identified.

Extracellular metals are sensed by one of three two-component systems in *Pseudomonas* species: CzcRS (104), CopRS (12) and ColRS (71, 72). Both CzcRS (activated by Zn^{2+}) and CopRS (activated by Cu^{2+}) induce expression of the heavy metal efflux pump CzcCBA while downregulating the OprD porin, leading to decreased carbapenem and imipenem uptake (12, 104). In *P. putida*, the ColRS system senses Zn^{2+}, Fe^{3+}, Mn^{2+} and Cd^{2+}, and mutants of *colR* and *colS* display lower tolerance to these metals (72). We have now determined Zn^{2+} to be an activating signal for ColRS in *P. aeruginosa* (Fig. 3.10 and 3.11). Additionally, the PA14 *colR* and *colS* mutants are more

sensitive to Zn^{2+} than wild-type, suggesting a role for the ColRS system in Zn^{2+} tolerance in *P. aeruginosa*. While the ColRS system is important for metal tolerance in *P. putida* and deletion of multiple genes in the ColRS regulon results in increased metal sensitivity, no individual ColRS-regulated gene has a major contribution to metal tolerance (72). It is likely that multiple genes in the *P. aeruginosa* ColRS regulon are involved in Zn^{2+} tolerance, which could explain why the $eptA_{Pa}$ isogenic mutant does not have any growth defect in media with Zn^{2+}.

Our demonstration of pEtN-modified lipid A via the ColRS system in response to Zn^{2+} (Fig. 3.12) reveals that lipid A remodeling in *P. aeruginosa* is more complex than previously realized. Extracellular Zn^{2+} specifically induces transcription of $eptA_{Pa}$ and not *arnT* through the ColR response regulator, demonstrating coordinated control over lipid A modifications. In addition to selectively inducing expression of $eptA_{Pa}$ Zn^{2+} downregulates *arnT* transcription by over 4-fold (Fig. 3.7B). This is interesting given that ArnT-mediated L-Ara4N modification typically plays a more significant role in virulence and antimicrobial peptide resistance than *eptA* in organisms possessing both modification enzymes (42, 43). While $eptA_{Pa}$ does not seem to be involved in metal or polymyxin resistance, there is likely an evolutionary reason for this targeted induction of $eptA_{Pa}$ transcription. Our findings demonstrate the tight control of *P. aeruginosa* lipid A modification systems, and suggest the need for further studies to better elucidate the mechanisms involved in outer membrane remodeling and its contribution to bacterial persistence and versatility.

Chapter 4: Conclusions and Future Directions

4.1 THE IMPORTANCE OF CONTINUED STUDY OF *P. AERUGINOSA* LIPID A MODIFICATIONS

4.1.1 Further characterization of *P. aeruginosa* lipid A remodeling systems

The underlying mechanisms responsible for *P. aeruginosa* virulence are continuously being examined in an attempt to improve treatment of *Pseudomonas* infections and improve patient prognosis. While *P. aeruginosa* outer membrane remodeling is just one of many factors that contribute to this organism's survival and pathogenesis, the interplay between lipid A and the host innate immune system make it a particularly important molecule to study. Our finding and characterization of functional LpxT and EptA enzymes as well as their ability to be regulated in response to specific environmental cues demonstrates the strict control of LPS remodeling in *P. aeruginosa.*

As discussed in Chapter 2, there is much we still do not understand about the biological role of the LpxT enzyme. Although additional studies are necessary, several ideas have been proposed regarding the purpose of LpxT including the potential to use a diphosphate group on lipid A as an energy source (30). Further, as LpxT uses as its substrate a carrier lipid involved in both peptidoglycan synthesis and LPS O-antigen translocation, it is possible that LpxT-dependent modification of lipid A helps to couple peptidoglycan and LPS biosynthesis (30, 86). The precise mechanism involved in regulation of LpxT activity in *P. aeruginosa* is also currently unknown. While no PmrR ortholog is present in the *P. aeruignosa* genome, it is possible that either the PhoPQ or

PmrAB system, which both respond to limiting Mg^{2+}, are involved in regulating LpxT activity (31).

In addition to LpxT, the significance of EptA and LpxO enzymes in *P. aeruginosa* remains unclear. Although pEtN addition to lipid A in organisms such as *H. pylori* and *N. gonorrhoeae* plays a critical role in CAMP resistance and virulence within a host (44, 47), the biological function of this modification in *P. aeruginosa* is yet to be elucidated. The role of hydroxylation of the lipid A secondary acyl chains by the LpxO enzymes in *P. aeruginosa* is also unknown. As an ortholog of VisP exists in *P. aeruginosa*, it is possible that VisP and the LpxO proteins interact and are involved in some sort of stress response, similar to the case in *S. enterica* (29). Future studies are necessary to determine whether these proteins interact and if so, what role they may play.

4.1.2 The role of *P. aeruginosa* lipid A remodeling systems in polymyxin resistance

One of the most critical aspects of *P. aeruginosa* pathogenesis that remains to be fully understood is the precise role of lipid A remodeling in polymyxin resistance. Polymyxin B and its closely related derivative, polymyxin E (or colistin) are cyclic CAMPS produced by Gram-positive *Bacillus* species (32). They are also commonly the drugs of last resort used to treat multi-drug resistant *P. aeruginosa* infections (54, 107). These cationic peptides bind to the phosphate groups of lipid A to enable penetration of the outer membrane and cell lysis (108). Lipid A remodeling is a critical factor leading to resistance. High levels of polymyxin and colistin resistance among CF clinical isolates is common, and leads to increased lung damage and worsened prognosis in patients

harboring such strains (107). Although modification of lipid A with L-Ara4N in organisms like *E. coli* and *S. enterica* plays a significant role in CAMP resistance (38, 39, 42), in *P. aeruginosa* the exact contribution of this modification is still not fully understood. Ernst and colleagues have reported that addition of L-Ara4N to *P. aeruginosa* lipid A alone does not explain the amount of resistance seen among highly resistant clinical isolates (53). Yet despite this, L-Ara4N modification is a characteristic of virtually all colistin-resistant clinical isolates. Further, deletion of L-Ara4N from the genome of polymyxin-resistant clinical isolates results in a significant decrease in resistance (109). It is thought that L-Ara4N can predispose *P. aeruginosa* to resist low levels of CAMPS and promote further resistance (53). The pharmacokinetics of these cationic peptides are extremely complex and still not well understood, and it is likely that subinhibitory CAMP concentrations could "prime" bacteria to induce other more robust mechanisms of resistance (53, 107).

Efforts have been made to better understand the regulatory systems that control lipid A remodeling and consequently polymyxin resistance in *P. aeruginosa*. A subset of CF patients treated with colistin are found to have extremely high (>512 mg/L) levels of colistin resistance, which is commonly associated with mutation or deletion of *phoQ* (109). A recent study sought to identify genes critical for the high levels of resistance seen in these *P. aeruginosa phoQ* mutants (54, 109). The CprRS and ColRS two-component systems were found to contribute significantly to polymyxin resistance in *phoQ* mutants; deleting *cprRS* or *colRS* resulted in an approximately 4-fold decrease in polymyxin resistance relative to the *phoQ* mutant (54). Perhaps most interestingly,

however, is that despite the increased polymyxin sensitivity in the Δ*phoQ*Δ*colRS* and Δ*phoQ*Δ*cprRS* mutants, they retain L-4AraN lipid A modification (54). This suggests that there are additional factors or mechanisms aside from L-4AraN responsible for polymyxin resistance, and that these factors are likely regulated by the ColRS and CprRS two component systems. The CprRS system has recently been identified and characterized in *P. aeruginosa* (69). Despite extensive characterization of the ColRS system in *P. putida* (97) and our recent investigation of its role in inducing $eptA_{Pa}$ gene expression, the precise role of this system in *P. aeruginosa* remains unknown. Further characterization of transcriptional targets of both systems and the biological functions of these genes is warranted to expand our understanding of *P. aeruginosa* CAMP resistance.

4.2 UNDERSTANDING THE ROLE OF LIPID A IN *P. AERUGINOSA* INFECTIONS

4.2.1 Characterization of the lipid A profile in chronological *P. aeruginosa* isolates from CF patients

It is important to apply the knowledge gained from studying *P. aeruginosa* lipid A modifications in laboratory strains to clinically relevant situations. Due to *P. aeruginosa*'s prevalence in chronic CF infections, the lung environment and its effect on growth and virulence must be considered. As described in Chapter 1, previous studies have demonstrated that there are CF-specific forms of lipid A, with constitutive modifications and physiological consequences (15, 34, 53). Changes in these CF-specific lipid A modifications do occur, however, depending on the stage or severity of CF

infection (25, 53). To date, these studies have only compared lipid A species from patients of different ages or with different types of infection, not from within the same patient. To better understand if these modifications are adaptations that occur over time within the same patient as opposed to host differences, studies should be done comparing lipid A species from chronic infection isolates of the same patient.

4.2.2 Characterization of lipid A changes within *P. aeruginosa* biofilms

Another question of interest is whether cells growing within surface-associated cell communities, or biofilms, express different forms of lipid A throughout. To this end, Haagensen and colleagues have shown that the distribution of polymyxin-tolerant *P. aeruginosa* cells varies within a biofilm, with only the cap-forming subpopulation of cells expressing the protective *arn* (*pmrK*) operon (110). This study tested this question by growing 'typical' surface-attached biofilms in citrate media. Carbon source can vary tremendously depending on the surrounding environment and can affect biofilm shape (110); it will therefore be important to further investigate the distribution of various lipid A subtypes within *P. aeruginosa* biofilms grown in various environmental conditions and with different carbon sources.

Addressing the distribution of lipid A modifications within biofilms will also need to be approached differently when looking at CF infection isolates. In the early stages of CF infection, *P. aeruginosa* grows as individual freely-swimming cells within the lung's surface fluid. During later stages of infection, however, the bacteria often switch to a biofilm mode of growth, forming rounded cell-to-cell attached groups known as

microcolonies (111–114). This change in growth is also associated with rapid deterioration of the lung (111). As biofilm-grown *P. aeruginosa* has been shown to express more inflammatory LPS than planktonically grown cells (115), it would be of interest to understand how the formation of microcolonies contributes to lipid A modification of *P. aeruginosa* in CF infections. Since microcolonies within the CF lung fundamentally differ from biofilms in that they are not attached to a surface, the effect of a more relevant growth media on *P. aeruginosa* lipid A structure remains to be examined (113, 116). Ideally, CF isolates should be cultured in CF sputum. Another option is to work with a medium that compositionally resembles sputum. To this end, synthetic cystic fibrosis medium (SCFM) has been developed, and has been shown to more closely mimic growth conditions found in the lung (117, 118). Future studies should begin to address the distribution of lipid A modifications in *P. aeruginosa* microcolonies formed in SCFM and/or CF sputum.

4.2.3 The role of lipid A modifications *in vivo*: the need for improved animal models

Perhaps the most important step toward better understanding the biological role of lipid A and the effects of various lipid A modifications will be the development of improved animal models for more accurate *in vivo* studies. For a number of years, there was a lack of an ideal animal model for the study of *P. aeruginosa* CF infection *in vivo.* While a number of models exist in which the animal exhibited some symptoms of CF, none of these models resulted in long-term, chronic bacterial infection as well as associated symptoms of the disease (111). One example is the *CFTR*-deficient mouse

model developed in 1992 (119, 120). While these mice did show elevated proinflammatory signaling, they did not spontaneously develop CF lung disease and also were able to clear even large doses of typical CF-associated pathogens without antibiotic treatment (121). Recently, *CFTR*-deficient and *CFTR* mutant pigs have been made (122, 123), and shown to spontaneously develop lung disease characteristic of CF (124). This promising new model will potentially be useful for testing the pharmacological efficacy of new drugs to treat CF infections (121).

Another critical flaw of animal models is the differential lipid A stimulation of TLR-4 in humans versus animal hosts. As discussed in Chapter 1, the shape and acylation pattern of lipid A affect its immunostimulatory properties, with hexa-acylated structures characteristic of *E. coli* and *P. aeruginosa* CF isolates showing the greatest levels of TLR-4 stimulation (125). Additionally, differences also exist in TLR-4 between species or even individuals within the same species that affect the biological response a given lipid A molecule will elicit (126). Although human TLR-4 robustly responds to hexa-acylated lipid A structures, it is not stimulated by penta-acylated species (126, 127). In contrast mouse TLR-4 responds strongly to both penta and hexa-acylated species, and thus does not respond to changes in the lipid A acylation pattern that might occur *in vivo* (126, 128). It is possible that this contributed to the failure of the mouse CF model to accurately simulate the pulmonary damage seen in human infection (121).

Using various *P. aeruginosa* isolates as TLR-4 agonists, an 82-amino acid hypervariable region in mouse TLR-4 was found to recognize both the hexa-acylated lipid A found in *Pseudomonas* chronic infection isolates and the penta-acylated form

found in acute infection and environmental isolates . Despite the clear difference in lipid A recognition between human and mouse TLR4, mouse studies attempting to look at the *in vivo* effects of various lipid A modifications have continued to be the norm. Hajjar and colleagues have now developed a transgenic humanized TLR4/MD2 line for more accurate *in vivo* studies of bacterial endotoxin (129). With this humanized mouse model, the molecular mechanisms related to pathogenesis within mammalian hosts can now be better understood.

Chapter 5: Materials and Methods

5.1 METHODS

5.1.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in Chapter 2 are listed in Table 5.1; strains and plasmids used in Chapter 3 are listed in Table 5.2. *E. coli* strains were grown in LB broth or agar (Difco) at 37°C. *P. aeruginosa* strains were grown on LB agar plates, and overnight cultures were routinely grown in LB broth at 37°C. Overnight *P. aeruginosa* cultures were diluted to an OD_{600} of ~0.05 in either LB broth or morpholinepropanesulfonic acid (MOPS)-buffered minimal medium (50mM MOPS, 93mM NH_4Cl, 43mM NaCl, 2mM KH_2PO_4) supplemented with 3.5μM $FeSO_4 \bullet 7H_2O$, 20mM sodium succinate, and 2mM or 0.02mM $MgSO_4$, as indicated. Chloramphenicol was used at a concentration of 30μg/mL for *E. coli*. Ampicillin or carbenicillin were used at a concentration of 100μg/mL or 300μg/mL for *E. coli* or *P. aeruginosa,* respectively.

For growth of *P. aeruginosa* in medium with added metals in Chapter 3, LB was used, which allowed metals to remain in solution. For the initial screen of lipid A modifications in Chapter 3, metal salts were added in the following concentrations: 2mM $ZnSO_4$, 0.2mM $CdSO_4$, 0.1mM $Ga(III)NO_3$, 0.2mM $FeSO_4$, 0.1mM $CoCl_2$, 2mM $CuSO_4$, 2mM $MnSO_4$, and 2mM $NiSO_4$. The highest concentration of metal that did not significantly reduce growth (defined as a >50% reduction in OD_{600}) in liquid medium was used, with the exception of Fe^{3+}, for which a lower, more physiologically relevant

concentration was used based on concentrations known to induce lipid A modification in other organisms (42).

5.1.2 DNA and RNA preparation

Before preparing *P. aeruginosa* genomic DNA from an overnight culture in LB broth, two washes with 0.1M NaCl were performed. Genomic DNA was prepared using the Easy-DNA Kit (Invitrogen). Total RNA was extracted from cells grown to an OD_{600} of ~0.6 using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. To eliminate residual DNA contamination, total RNA was treated with DNase from the RNase-Free DNase Set (Qiagen). cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

5.1.3 Recombinant DNA methods

Plasmid DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). Chromosomal DNA for insertion into plasmid constructs was amplified using either the DNA polymerase *PfuTurbo*^{R} (Stratagene) or Takara *Ex Taq* (Takara). PCR products were separated on an agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). All primers were purchased from Integrated DNA Technologies; primers used in Chapter 2 are listed in Table 5.3, and those used in Chapter 3 are listed in Table 5.4. Restriction endonucleases, T4 DNA ligase, and Antarctic Phosphatase used in this study for generation of plasmid constructs were purchased from New England Biolabs and used according to the manufacturer's instructions.

5.1.4 Generation of chromosomal gene deletion mutants

In-frame, markerless gene deletions were generated in *P. aeruginosa* by homologous recombination using the suicide plasmid pEX18Gm (130). ~1Kb DNA fragments flanking the target gene up or downstream were amplified using primers listed in Tables 5.3 and 5.4. An assembly PCR was then carried out to stitch together these flanking regions. Assembly PCR fragments were digested with restriction endonucleases EcoRI and HindIII, and ligated into pEX18Gm. The suicide plasmid constructs, pEX18-*lpxT*del and pEX18*arnT*del for Chapter 2, and pEX18-*eptA*del, pEX18-*colR*del, and pEX18-*colS*del for Chapter 3 were introduced into *P. aeruginosa* via conjugation with *E. coli* strain SM10 (131). Deletion mutants were then screened for as described previously (130). Briefly, the gentamycin resistance cassette in pEX18 allows for selection of single recombinants, and a *sacB* gene confers sensitivity to growth on sucrose to allow selection of double crossover recombinants. Deletions were confirmed by PCR.

5.1.5 Plasmid constructs

5.1.5.1 Constructs generated in Chapter 2

Complementation of W3110Δ*lpxT* was achieved by expressing either $lpxT_{Ec}$ or $lpxT_{Pa}$ in the low-copy number plasmid pWSK29 (132), and PA14Δ*lpxT* was complemented by expressing $lpxT_{Pa}$ in the medium-copy number plasmid pEX1.8 (133). For generation of both constructs, the $lpxT_{Pa}$ gene and RBS were amplified using primers PA*lpxT*F and PA*lpxT*R (Table S2), digested with EcoRI and HindIII, and ligated into

vector cut with the same restriction endonucleases to yield p*lpxT*$_{Pa}$ and pEX*lpxT*$_{Pa}$. Constructs were confirmed by sequencing.

5.1.5.2 Constructs generated in Chapter 3

To construct pPA14_39020 (pAC*eptA*$_{Pa}$), pPA14_58610 and pPA14_21210, each gene was amplified along with the native RBS and cloned into the medium copy vector pACYC184 using EcoRV and SalI restriction endonucleases. For generation of p*eptA*$_{Pa}$, p*colR*, p*pmrA* and p*phoP*, each gene and its native RBS were amplified and digested with EcoRI and HindIII to clone into pEX1.8. All constructs were confirmed by sequencing. The *eptA*$_{Pa}$ and *colR* genes were amplified along with their native promoters and cloned into the medium copy vector pEX1.8 (133) by digestion with SalI or SalI and HindIII, respectively, generating p*eptA*$_{nprom}$ and p *colR*$_{nprom}$. For generation of p*colS*$_{nprom}$, the *colRS* promoter was first amplified and cloned into pEX1.8 by digestion with BamHI and EcoRI. The *colS* coding sequence was then amplified and cloned into pEX1.8 (containing the *colRS* promoter) with EcoRI and HindIII.

5.1.6 Isolation and analysis of labeled lipid A

Cultures from overnight growth were diluted to an OD_{600} of ~0.05 in 5mL of fresh medium (as indicated) and labeled with 2.5μCi/mL $^{32}P_i$ (Perkin-Elmer). Cells were harvested at an OD_{600} of 0.8-1.0, and lipid A was isolated by mild acid hydrolysis as previously described (41, 82). $^{32}P_i$-labelled lipid A species were spotted on a TLC plate at ~5,000 cpm per lane (10,000 cpm for *E. coli*), and analyzed in a solvent system

consisting of 50:50:16:5 (v/v) of chloroform, pyridine, 88% formic acid, and water, respectively. TLC plates were dried, exposed to a phosphor screen overnight and imaged using a phosphor-imager (BioRad PMI).

5.1.7 Large scale lipid A isolation and MALDI-TOF mass spectrometry

Large scale 250mL-1L cultures were grown at 37°C to an OD_{600} of ~1.0 in the medium indicated. Lipid A was prepared by mild acid hydrolysis, washed and dissolved in chloroform/methanol/water (2:3:1, v/v), as described (83). Samples were then applied to a DEAE cellulose column, washed with chloroform/methanol/water (2:3:1, v/v), and eluted as separate fractions using chloroform/methanol/increasing concentrations of aqueous ammonium acetate (60mM, 120mM, 240mM, 480mM) (2:3:1, v/v), as described previously (83, 134). Typically, hydrophilic or monophosphorylated species fractionate at lower ammonium acetate concentrations (flow-through, wash, 60 or 120mM elution fractions), while more hydrophobic, unmodified or phosphate-modified species fractionate at the highest concentration, 480mM ammonium acetate. MALDI-TOF mass spectrometry was performed as previously described using a MALDI-TOF/TOF mass spectrometer (ABI 4700 Proteomics Analyser) (83).

5.1.8 ESI and UVPD mass spectrometry

Lipid A was isolated and prepared as described above for MALDI-TOF analysis. All mass spectrometry experiments were executed on a Thermo Scientific Orbitrap Elite mass spectrometer (Bremen, Germany) modified to perform ultraviolet photodissociation

(UVPD). The mass spectrometer was equipped with a 193-nm Coherent ExciStar XS excimer laser (Santa Clara, CA) and operated in the negative ion mode using a previously described set-up (135). Briefly, solutions containing 1-5 μM lipid A in 50:50 methanol/ chloroform were directly infused using an electrospray ionization (ESI) source at a flow rate of 3 μl/min. The ESI voltage was set to 4 kV. UVPD mass spectra were collected using 10 laser pulses per spectrum (at 4-5 mJ/pulse) and were interpreted as described previously (136).

5.1.9 Quantitative and Semi-quantitative PCR methods

Primers for semi-quantitative and quantitative PCR (qPCR) were designed using the Primer-BLAST tool (NCBI) and are listed in Tables 5.3 and 5.4. Semi-quantitative PCR performed in Chapter 3 was executed by amplifying cDNA obtained from samples cultured in the conditions or with the p*pmrA*, p*phoP* and p*colR* expression constructs as indicated, using primers specific for $eptA_{Pa}$ or *clpX* as a reference gene (84). qPCR was performed in a OneStep thermocycler (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions, as described previously (31, 137).

5.2 STRAINS, PLASMIDS AND PRIMERS USED

Table 5.1. Stains and plasmids used in Chapter 2

Strain or Plasmid	Description	Reference
Strains		
E. coli		
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZΔM15*::Tn10 (Tet^r)]	Stratagene
W3110	Wild-type, F^-I^- rph-1 INV(*rrnD, rrnE*)1 *rph-1*	*E. coli* Genetic Stock Center (Yale)
W3110Δ*lpxT* (MST01)	W3110, Δ*lpxT*	(42)
SM10	*thi-1 thr leu tonA lacY supE recA*:: RP4-2-Tc::Mu (Kan^r)	(de Lorenzo and Timmis, 1994)
P. aeruginosa		
PA14	Wild-type, UCBPP-PA14	(138)
PA14Δ*lpxT*	PA14, Δ*lpxT*	This study
PA14Δ*arnT*	PA14, Δ*arnT*	This study
Plasmids		
pWSK29	Low copy vector, T7 and T3 RNA polymerase promoters,	(132)

Table 5.1 (continued)

Strain or Plasmid	Description	Reference
	ampicillin resistance	
p*lpxT*$_{Ec}$	pWSK29 containing *E. coli lpxT* coding sequence and ribosome binding site (RBS)	(42)
p*lpxT*$_{Pa}$	pWSK29 containing *lpxT*$_{Pa}$ coding sequence and RBS	This study
pEX18Gm	Suicide (gene replacement) vector, *oriT*+ *sacB*+, pUC18 MCS, gentamycin resistance	(130)
pEX18Gm:*lpxTdel*	pEX18Gm containing assembly PCR product of 1Kb upstream and downstream flanking regions of *lpxT* coding sequence	This study
pEX18Gm:*arnTdel*	pEX18Gm containing assembly PCR product of 1Kb upstream and downstream flanking regions of *arnT* coding sequence	This study
pEX1.8	Medium copy vector, pEX1 carrying *ori* (*P. aeruginosa*) as 1.8-kb (133)(132)(131)(129)(127)(126)(125)(124)(123)(122)(5)*Pst*I fragment from pRO1614 in *Sty*I site	(133)
pEX*lpxT*$_{Pa}$	pEX1.8 containing *lpxT*$_{Pa}$ coding sequence and RBS	This study

Table 5.2. Strains and plasmids used in Chapter 3.

Strain or plasmid	Description	Reference
Strains		
E. coli		
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZ*Δ*M15*::Tn10 (Tet^r)]	Stratagene
W3110	Wild-type, F^-I^- rph-1 INV(*rrnD, rrnE*)1 *rph-1*	*E. coli* Genetic Stock Center (Yale)
W3110Δ*eptA* (CH030)	W3110, Δ*eptA*	(42)
BN2	K-12 BW25113, Δ*pagP*, Δ*lpxT*, Δ*eptA*, Δ*lpxM*	(4)
SM10	*thi-1 thr leu tonA lacY supE recA*:: RP4-2-Tc::Mu (Kan^r)	(131)
P. aeruginosa		
PA14	Wild-type, UCBPP-PA14	(138)
PA14Δ$eptA_{Pa}$	PA14, Δ$eptA_{Pa}$	This study
PA14Δ*colR*	PA14, Δ*colR*	This study
PA14Δ*colS*	PA14, Δ*colS*	This study
Plasmids		
pACYC184	Low copy-number cloning vector, tet^R, cam^R	Novagen
pPA14_58610	pACYC184 containing PA14_58610 coding sequence and RBS	This study
pPA14_21210	pACYC184 containing PA14_21210 coding sequence and RBS	This study
pPA14_39020 (pAC$eptA_{Pa}$)	pACYC184 containing PA14_39020 ($eptA_{Pa}$) coding sequence and RBS	This study
p$lpxE_{Fn}$	pWSK29 containing *F. novicida lpxE*	(49)
p$lpxF_{Fn}$	pWSK29 containing *F. novicida lpxF*	(50)
pEX1.8	Medium copy-number cloning vector, pEX1 carrying *ori* (*P. aeruginosa*) as 1.8-kb (133)(132)(131)(129)(127)(126)(125)(124)(123)(122)(5)(7)(6)(5)*Pst*I fragment from pRO1614 in *Sty*I site	(133)
p$eptA_{Pa}$	pEX1.8 containing $eptA_{Pa}$ coding sequence and RBS	This study

Table 5.2 (continued)

Strain or plasmid	Description	Reference
p*eptA*$_{\text{nprom}}$	pEX1.8 containing *eptA*$_{\text{Pa}}$ coding sequence and native promoter	This study
p*pmrA*	pEX1.8 containing *pmrA* coding sequence and RBS	This study
p*phoP*	pEX1.8 containing *phoP* coding sequence and RBS	This study
p*colR*	pEX1.8 containing *colR* coding sequence and RBS	This study
p*colR*$_{\text{nprom}}$	pEX1.8 containing *colR* coding sequence and native promoter	This study
p*colS*$_{\text{nprom}}$	pEX1.8 containing *colS* coding sequence and native promoter	This study
pEX18Gm	Suicide (gene replacement) vector, *oriT*+ *sacB*+, pUC18 MCS, gentamycin resistance	(130)
pEX18Gm:*eptA*del	pEX18Gm containing assembly PCR product of 1Kb upstream and downstream flanking regions of *eptA* coding sequence	This study
pEX18Gm:*colR*del	pEX18Gm containing assembly PCR product of 1Kb upstream and downstream flanking regions of *colR* coding sequence	This study
pEX18Gm:*colS*del	pEX18Gm containing assembly PCR product of 1Kb upstream and downstream flanking regions of *colS* coding sequence	This study

Table 5.3. Primers used in Chapter 2.

Primer	Sequence (5'-3')	Application
PA*lpxT*F	CTGAATTCATAGGGGGAAACGATTTTATGGATAATGC	*cloning* $lpxT_{Pa}$ into pWSK29 and pEX1.8
PA*lpxT*R	GTCCGCGACTCGGCCTGAAAGCTTGC	*cloning* $lpxT_{Pa}$ into pWSK29 and pEX1.8
*lpxT*del-outF	GCAGGTAGTAAGCCTCGGCGGAACCTACATG	cloning upstream *lpxT* flanking region, assembly PCR for *lpxT* deletion
*lpxT*del-outR	GTGCTGCTCGGCTCCAAGGCGCTGATCG	cloning downstream *lpxT* flanking region, assembly PCR for *lpxT* deletion
*lpxT*del-inF	CGATAGGGGGAAACGATTTTATGGATAATGCCTCTGACTCGGCCTGAGGCCATCC	cloning downstream *lpxT* flanking region
*lpxT*del-inR	GGATGGCCTCAGGCCGAGTCAGAGGCATTATCCATAAAATCGTTTCCCCCTATCG	cloning upstream *lpxT* flanking region
*arnT*del-outF	TGCTCGACTGGCAGCCCAC	cloning upstream *arnT* flanking region, assembly PCR for *arnT* deletion
Primer	**Sequence (5'-3')**	**Application**
*arnT*del-inF	CAGACCTGGTCGCTGCTGCTGACCTGGTCCTG	cloning downstream *arnT* flanking region
*arnT*del-inR	CAGGATCGCCAGGACCAGGTCAGCAGCAGCGACCAGGTCTG	cloning upstream *arnT* flanking region
PA*clpX*F	AAGAAGGTTCTGGCGGTAGC	qPCR analysis of *clpX* expression
PA*clpX*R	ATGTTCTCGACATCCTCGCC	
PA*arnT*F	GGCTATGCCAACCTCGACCC	qPCR analysis of *arnT* expression
PA*arnT*R	GCGAGGAAGCCCTTGGTCAG	

Table 5.3 (continued)

PA*lpxT*F	CTGACCTTCGGCTTCATCGT	qPCR analysis of *lpxT*
PA*lpxT*R	TGGAGCGGTCCTTGATTTCC	expression

Table 5.4. Primers used in Chapter 3.

Table 5.4 (continued)

Primer	**Sequence (5′-3′)**	**Application**
5′ PA14_58610	GCGATATCCCATCGAACGAGGCTA TCGTGTC	Used to clone PA14_58610 and native RBS into pACYC184
3′ PA14_58610	CGCGGATCCGCGTCAGCCTTCGTTC GGCTG	
5′ PA14_21210	CGAGCGATATCTCAGGAGTTGCTTC AATGGGT	Used to clone PA14_21210 and native RBS into pACYC184
3′ PA14_21210	CGCGGATCCTCACTGGGCTGCCCTG GG	
5′ PA14_39020	CGAGCGATATCCAGTGAAGATCCG TGCCCATG	Used to clone PA14_39020 (*eptA*$_{Pa}$) and native RBS into pACYC184
3′ PA14_39020	CGATGTCGACTCAGGAAGCCGGCG GCTCCT	
5′ PA*eptA*	CGAGCGAATTCCAGTGAAGATCCG TGCCCATG	Used to clone *eptA*$_{Pa}$ into pEX1.8
3′ PA*eptA*	CGATAAGCTTTCAGGAAGCCGGCG GCTCCT	
5′ *eptA*outdel	GAGAGCTCGAAGCGTCCGACGGTG TCCAG	Used to clone upstream *eptA*$_{Pa}$ flanking region and for assembly PCR to generate pEX18Gm:*eptA*del
3′ *eptA*outdel	GAGGATCCGTCCCGATGAACCACC CGCA	Used to clone downstream *eptA*$_{Pa}$ flanking region and for assembly PCR to generate pEX18Gm:*eptA*del
5′ *eptA*indel	GATCCGTGCCCATGTCGAAAGCCG AGGAAGCGTCTGGCCAGGAG	Used to clone downstream *eptA*$_{Pa}$ flanking region
3′ *eptA*indel	CTCCTGGCCAGACGCTTCCTCGGCT TTCGACATGGGCACGGATC	Used to clone upstream *eptA*$_{Pa}$ flanking region
5′ PA*eptA*$_{nprom}$	ACGCGTCGACGAAATTCACAGAAG GGTTTCCCG	Used to clone *eptA*$_{Pa}$ and native promoter (nprom) into pEX1.8
3′ PA*eptA*$_{nprom}$	CAAGACGTCGACTCGCCGGGTCGT ATCAGGAA	
5′ PA*pmrA*	CGGAATTCATGAGAATACTGCTGG CCGAGGACGACCT	Used to clone *pmrA* into pEX1.8
3′ PA*pmrA*	CCCAAGCTTTCAGGGCGCCGGCTG GTC	

Table 5.4 (continued)

Primer	Sequence (5′-3′)	Application
5′ PA*phoP*	CGGAATTCATGAAACTGCTGGTAGTGGAAGACGAGG	Used to clone *phoP* into pEX1.8
3′ PA*phoP*	CCCAAGCTTTCACCGGCAGCGCTCGGTG	
5′ PA*colR*	CGGAATTCATGCGAATACTGGTGGTCGAAG	Used to clone *colR* into pEX1.8
3′ PA*colR*	CCCAAGCTTTTATACTCCATTCGGCTCCTCC	
5′ *colR*outdel	GTTCTACATGAAGGTGCCCATCGAGTTCGG	Used to clone upstream *colR* flanking region and for assembly PCR to generate pEX18Gm:*colR*del
3′ *colR*outdel	CGCAGCAGTGGCGCGTTGAACTG	Used to clone downstream *colR* flanking region and for assembly PCR to generate pEX18Gm:*colR*del
5′ *colR*indel	GGACATGCGAATACTGGTGGAGCCGAATGGAGTATAAGC	Used to clone downstream *colR* flanking region
3′ *colR*indel	GCTTATACTCCATTCGGCTCCACCAGTATTCGCATGTCC	Used to clone upstream *colR* flanking region
5′ *colS*outdel	CGGAATTCCGAGGAACAGCCAGGGGTTGAATGGAC	Used to clone upstream *colS* flanking region and for assembly PCR to generate pEX18Gm:*colS*del
3′ *colS*outdel	CGGGATCCCACGCGAATACCAAGCGACTCGAAGGC	Used to clone downstream *colS* flanking region and for assembly PCR to generate pEX18Gm:*colS*del
5′ *colS*indel	GAGGAGCCGAATGGAGTATAAGCTCGATGTTGCTTGACGA	Used to clone downstream *colS* flanking region
3′ *colS*indel	TCGTCAAGCAACATCGAGCTTATACTCCATTCGGCTCCTC	Used to clone upstream *colS* flanking region
5′ PA$colR_{nprom}$	ACGCGTCGACCGATCACCAGTTGCTTGAC	Used to clone *colR* and native promoter (nprom) into pEX1.8
3′ PA$colR_{nprom}$	GAGGAGCCGAATGGAGTATAAAAGCTTGC	
5′ PA$colS_{nprom}$	CGCGGATCCCGATCACCAGTTGCTTGAC	Used to clone *colRS* native promoter (nprom) into pEX1.8
3′ PA$colS_{nprom}$	GCGAATTCGTCCCACTCCTTCGCAGGA	

Table 5.4 (continued)

Primer	**Sequence (5′-3′)**	**Application**
5′ PA*colS*	GCGAATTCATGGAGTATAAGCAGA GCCTCGC	Used to clone *colS* into pEX1.8
3′ PA*colS*	GGAAGCTTTCAAGCAACATCGAGT AAAACTTCGAACC	
5′ q*clpX*	AAGAAGGTTCTGGCGGTAGC	qPCR analysis of *clpX*
3′ q*clpX*	ATGTTCTCGACATCCTCGCC	transcription
5′ q*eptA*	TGCCCTGCATGTTCTCCAAC	qPCR analysis of *eptA*
3′ q*eptA*	GATCCTTGCTCTCGCTCAGG	transcription
5′ q*arnT*	GGCTATGCCAACCTCGACCC	qPCR analysis of *arnT*
3′ q*arnT*	GCGAGGAAGCCCTTGGTCAG	transcription

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